UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Advances in the study of inflammation biomarkers in the saliva of pig.

Avances en el estudio de biomarcadores de inflamación en saliva de cerdo.

Dª. María José López Martínez

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Autora: Dª. María José López Martínez

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Dª. Silvia Martínez Subiela

UNIVERSIDAD DE MURCIA

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Aprobado por la Comisión General de Doctorado el 19-10-2022

D./Dña. María José López Martínez

doctorando del Programa de Doctorado en

Ciencias veterinarias

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AGRADECIMIENTOS

Cuando empecé esta Tesis Doctoral, no sabía muy bien dónde me estaba metiendo, supongo que como cualquier otro doctorando. ¿Dónde le enseñan a uno a "hacer ciencia"? Porque a lo largo de nuestra vida académica nos hemos ido empapando con ciertas nociones (al fin y al cabo, el método científico está presente en casi todo lo que aprendemos), pero cuando uno llega al doctorado es cuando realmente empieza a entender los códigos necesarios para crearla. El máximo exponente de la actividad investigadora serían los artículos científicos, los "papers", donde se plasman los resultados obtenidos, de la forma más objetiva y separada del "yo" posible. Pero ¿dónde queda todo aquello que sucede en el laboratorio mientras hacemos los experimentos? ¿Y lo que ocurre tras colgar la bata y llegar a casa los días en los que no salen las cosas bien? Algo que ocurre la mayoría del tiempo, porque si no, no se llamaría "investigación"…

Pues, como dejó grabado en mi mente Rosalind Franklin, "la ciencia y la vida cotidiana no pueden ni deben estar separadas". Es imposible separar la razón del corazón. Todas las personas que han estado a mi lado estos años, en lo personal y en lo profesional (aspectos en los que frecuentemente se produce una "fusión", similar a la de los anticuerpos monoclonales), me han hecho sentir cómoda, feliz, arropada y, en definitiva, querida. No es algo que se pueda plasmar en un apartado convencional de "Material y métodos", pero aquí sí me voy a tomar la libertad de explayarme y poder dar su lugar a todos los que, durante estos años, han formado parte de mi vida y, por tanto, también de la ciencia y el corazón que hay detrás de esta Tesis Doctoral.

A los ejes centrales de esta tesis: mis directores, Cerón y Silvia, que estuvieron ya ahí desde los tiempos de mi TFG, me abrieron la oportunidad de empezar este camino, y me transmitieron su pasión y entrega por la investigación. Durante toda la tesis han sido parte activa en todos los aspectos, ya sea en la planificación, discusión de los resultados, y no menos importante, de los "no resultados" (o lo que es lo mismo, "rayadas"). Me quedo con vuestra paciencia y resiliencia infinita, que sin duda han sido un gran aprendizaje para mí. Sin vosotros, vuestro trabajo y apoyo continuo, nada de esto sería posible. Por supuesto, tampoco sería posible sin la Universidad de Murcia y sin la oportunidad que me brindó la Fundación Séneca, entidad financiadora de esta tesis, a través de los contratos predoctorales FPI/19. GRACIAS.

También a otros miembros del profesorado, como Juan Diego e Ignacio, con los que hice TFG y alumnado interno, respectivamente, y gracias a los cuales empecé en el departamento; a Luis Bernal, mi mayor maestro en clínica, que me permitió aprender muchísimo junto a su maravilloso equipo durante varios años; a Fernando Tecles y Asta, que durante estos años de doctorado siempre me han ayudado en todo lo posible; y a Guillermo Ramis y Silvia Martínez Miró, que han sido uno más de los nuestros en muchos muestreos. GRACIAS.

También a todo el personal de la Universidad de Murcia que hace posible que podamos acceder a recursos esenciales para el desarrollo de nuestra actividad investigadora; con especial mención al personal que ha formado y/o forma parte del SACE, que siempre han estado ahí para echarnos una mano: Juana, Toñi, Silvia, Pilar, Mª Jesús y Vero. GRACIAS.

Por supuesto, a todo el grupo que formamos Interlab-UMU, aunque a algunos os pueda ver menos diariamente por motivos "físicos", pero con los que empecé (especialmente con Isa y sus clases magistrales de Olympus) y que siempre me habéis seguido dando vuestra ayuda y cariño dentro y fuera del trabajo, desde Susana, patrona del LAB4 y "telemáticamente" del pleiades, (¡qué haríamos sin ti!), pasando por mi casi compi de tesis, Luis Pardo; hasta Belén, Fran y Carmen, que trabajan incansablemente para que todo vaya siempre "rodado". GRACIAS.

Especialmente, a todos mis compañeros del pleiades, que son mucho más que co-autores de mis artículos. Es una suerte, de verdad, haber trabajado con vosotros codo con codo, y cerveza con cerveza (bueno, en mi caso, agua…) durante todo el camino. Damián, fuiste el primero que me llevó de la mano en mis inicios aquí, con el TFG, y que periódicamente ha ido chequeando que me fuera todo bien; tu mítica pregunta "¿Qué? ¿Cómo va esa tesis?", siempre ha dado lugar a muchos momentos de desahogo y reflexión. Ana Cantos, empezaste a resultarme familiar también durante la época de mi TFG; sin apenas conocerme, siempre me animabas cada vez que nos cruzábamos por el LAB4, y tu defensa de Tesis Doctoral, junto con la de Alberto, fue la primera experiencia que tuve con el proceso en el que yo ahora me encuentro (disimularé que tras ese día estuve a punto de salir corriendo…). De igual modo, con el TFG te conocí a ti, Mariló: desde el principio me ayudaste incansablemente con todo lo que necesité, y así ha seguido siempre; incluso con lo que más odias, ¡la proteómica! Marina, eres la persona más bondadosa y madrugadora (bueno, ahí ahí con Luisito) del plei; siempre que llegaba estabas ahí, con una sonrisa y medio AlphaLISA hecho (reconozco que cuando se me hacían a mí las 15h para poner mi placa, siempre pensaba "mañana llego a la misma hora que Marina"… nunca sucedió). Junto a Sandra, ambas fuisteis mis primeritas mentoras ya empezando la etapa del doctorado. Os debo todo lo que sé sobre anticuerpos, pero también sobre lo más importante: el momento de almorzar, "tomar aire" y reír juntas, algo a lo que sin duda hay que hacer hueco (a veces con dificultad, a veces digo que me quedan 10 minutos pero en realidad se hacen 20…). Lorena, lo primero que supe de ti es que también eras de Molina como yo y, entre nosotras, sabemos que eso puede ser muy bueno o muy malo…, en tu caso, excelente: de ti he aprendido una positividad contagiosa (y no me he sentido tan sola en mi idea de que la multitarea suele acabar mal). Ana Huertas, tú también me enseñaste a sobrellevar mejor mis errores, con tus tiernos "Ayy nooo!! Creo que la he liado!!" y a enfrentarme a nuevas experiencias, como mi primera estancia en Ciudad Real, a la que fuimos juntas en plena pandemia, y que aun así no pudo ir mejor. Camila, siempre has tenido

los consejos y las palabras de cariño que más han logrado arroparme, he aprendido muchísimo de ti, y has llevado el sentido de "familiaridad" en el plei a otro nivel. Luisito, empezamos la tesis juntos y parecía que íbamos a acabarla juntos pero al final no (yo ya me he quitado esto de enmedio, pero tú no, ¡ahí la llevas!); aun así para mí nuestro camino ha sido conjunto y las mañanas no serían lo mismo sin tu motivación musical y sin tu "¡Niñoos! ¡A desayunar!". Alba, me gustaría aprender de tu generosidad, siempre atenta de ayudarnos a todos (aunque a veces te deberías moderar, ¡que no llegas ni a lo tuyo!); ¿qué sería del "team tardes" sin las risas que nos echamos siempre? María, has sido la última en empezar el doctorado con nosotros, pero con muy bien pie, desde el principio siguiendo las buenas costumbres (es decir, los almuerzos y cenitas, por supuesto). Gregorio y Adrián, de vosotros me encantaría aprender vuestra capacidad de adaptación y vuestro sentido del humor, no sé cuántas técnicas diferentes os he visto hacer a lo largo de mis años de doctorado, pero desde luego darían para otro doctorado seguro. Y de Franky, la más reciente incorporación al pleiades, me gustaría aprender tu paciencia y tu serenidad procesando muestras de cadáveres de animales que… en fin, hay que olerlas para entenderlo. Finalmente, el último por una buena razón, haciendo honor a los papers, Alberto, mi compadre y mi "director honorífico": has sido esencial, especialmente en la segunda etapa de mi tesis. Creo que nunca te lo he dicho, pero tu humildad es lo que más admiro de ti, pues sacándome tres pueblos de experiencia (habiéndote visto defender tu tesis cuando yo era, como tú mismo dirías, "una mindundi"), te has convertido en mi guía de la forma más cercana y risueña posible, haciéndomelo todo extremadamente sencillo. Siempre te las apañas para sacarme una sonrisa, ya sea en el laboratorio, en un paseo con el Nico, un roadtrip a Zaragoza, una comida o un futbolín. A todos vosotros, no puedo más que daros un millón de GRACIAS.

A todos los investigadores y veterinarios con los que me he cruzado en el camino, desde los que me han ayudado en mil recogidas de muestras, como Aída, Elena o Raquel, pasando por todos los autores con los que he colaborado en papers a lo largo de estos años, hasta los que me han acogido con los brazos abiertos allá donde he ido a hacer estancias. Entre estos últimos, agradecer en especial al equipo del IREC en Ciudad Real, capitaneado por Marinela, con tu eterna sonrisa y tus múltiples respuestas para absolutamente todo; al grupo de porcino de TEAGASC en Cork, encabezado por Edgar Manzanilla, con el que compartí momentazos muy "random" de los que me acordaré siempre; y al grupo de Fisiología Animal de Évora, dirigido por Elsa Lamy, eternamente dispuesta a ayudar y a practicar con nosotros el "portuñol". GRACIAS.

A mis animales, porque sin ellos no habría estudiado veterinaria y no estaría hoy aquí, ni habría descubierto una parte esencial de mi ser. En vuestros ojos he encontrado siempre la verdadera inocencia, la lealtad incondicional, y la motivación para saber cómo poder cuidaros lo mejor posible… desde mis hámsters (Benito, Godofredo, Salustiano, y Mateo; no, no había

nombres más normales…), pasando por mis cobayas (Cobi y Mancha; tampoco había nombres más evidentes…), hasta mis actuales periquitos "enamoraos" (Blue y Pepa); e incluso mi futuro perro, que aun ni lo conozco, pero pienso en él desde que tengo uso de razón. GRACIAS.

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A mi familia. A mis padres, Pedro y María José, que me habéis enseñado las bases de todo lo que sé, que me habéis apoyado siempre y me habéis proporcionado todo lo que ha estado en vuestra mano para que pueda llegar a conseguir lo que me proponga. Nos habéis llevado siempre a vivir mil aventuras (no hay sitio que recuerde en el que no hayamos estado con la caravana), a mí y David, mi hermano, "el neni": contigo he inventado siempre mil tonterías con las que reírnos y que sólo entendíamos nosotros; desde pequeña me "corrompiste" en las buenísimas costumbres de quedarnos despiertos a escondidas hasta las tantas y escaparnos de noche al salón a ver Buenafuente, algo que con los años se convirtió en acostarnos ya bien salido el sol (is that the sun?) tras pasar la noche editando nuestros cortos. Todas estas experiencias han contribuido enormemente a despertar mi imaginación y mi curiosidad por el mundo, y no puedo más que

agradecéroslo. A mis “abueicos”, Miguel y Mary, desde aquellos imborrables veranos cuando nos despertábamos con las composiciones de piano del abuelo, nos zampábamos una sopa de arroz de la abuela, y grabábamos cosas juntos, incluyendo aquellos momentos en los que aún no sabía leer, pero me parecía algo tan fascinante que me lo inventaba. Todo lo que me habéis inculcado forma parte de mí, y si en algún momento se me olvida, tengo la suerte de que puedo “tirar de hemeroteca”. A mis otros abuelitos que ya no están, José María y Basilisa, en cuyo patio podíamos pasar horas montando a la bici, haciendo nuestros fuertes, o incluso empezando mi formación veterinaria cuidando a “gatitos” tuertos o murciélagos rescatados. A todos mis tíos: Mamen, siempre un ejemplo en lo académico y en no rendirte nunca; Víctor, con tus míticas “caídas” que nos levantan a todos; Paqui, con tu positividad y generosidad altamente contagiosas; Migue, con tu apoyo incondicional frente a todo lo que hago; Ilu, con tu maravilloso sentido del humor; Maria Consuelo, con tu naturalidad embaucadora; Jose, al que tantas veces hacíamos reír David y yo en casa de los abuelitos… y primos: Emma, ya eres toda una “pana” más; Miriam, mi compañera tronchante; Miguel, el más cabecilla loca; Roci y Lucas, los más fiesteros y atentos con todo lo que hemos necesitado siempre; y Rubén y Lorena, siempre deshechos en halagos con nosotros. A mis suegros María y Antonio, y a mi cuñada María, pues ya sois parte de mi familia y yo de la vuestra; desde el primer día que me conocisteis me tratasteis como a una hija y una hermana más, con todo vuestro cariño y apoyo. A todos los que, con el paso de los años, habéis ido ampliando la familia, que tiende a infinito: MJ, nuestra “pitonisa” de confianza, y Manu, el último fichaje del maravilloso círculo de “los panas”; así como toda la familia y amigos de Lucas, que os apuntáis siempre a todos los jolgorios posibles. TODOS sois mucho más que familia, y ya sea en comidas, en excursiones, en scape rooms o en casas rurales, pasar tiempo con vosotros es siempre un lujo. Todos y cada uno de vosotros habéis confiado y creído siempre en mí y en mis posibilidades, y me habéis dado siempre mucha fuerza para continuar. GRACIAS.

A Jose, que es la mayor expresión del sentimiento de familia: la que uno elige formar. Desde que llegaste, trajiste la luz con la máxima intensidad posible. Has presenciado todas y cada una de mis caídas a lo largo de este camino, y no has dudado nunca en saltar la barrera y tirarte en plancha para cogerme y levantarme. Eres la eterna sonrisa, la ternura, el corazón, el alma; quedan pocas personas como tú, y yo tengo la inmensa suerte de tenerte a mi lado. Me has vuelto a enseñar todo desde cero: estar contigo es calma, libertad, un soplo de aire fresco, confianza ciega, felicidad. Me has sabido entender como nadie, y me has acompañado a todas partes a pesar de tener muchos miedos, que hemos ido superado juntos. Aún nos queda mucho por caminar, siempre hacia adelante, siempre de la mano. GRACIAS.

De nuevo, a todos: GRACIAS. Todos formáis parte de esta tesis, de un modo u otro. Solo espero poderos haber aportado aunque sea un poco de todo lo que me habéis regalado a mí.

"La imaginación es la Facultad del Descubrimiento.
Es lo que penetra en los mundos nunca vistos a nuestro alrededor:
los mundos de la ciencia"

Ada Lovelace, 1841

INDEX

DOCTORAL THESIS AS COMPENDIUM OF PUBLICATIONS

In accordance with the authorization of the directors of the PhD Thesis and the Academic Commission responsible for the Veterinary Sciences PhD Program, this PhD Thesis is presented as a compendium of nine studies previously published. Therefore, the PhD Thesis is composed of the following references:

I. **López-Martínez, M.J.,** Franco-Martínez L., Martínez-Subiela S., Cerón J.J. (2022). Biomarkers of sepsis in pigs, horses and cattle: from acute phase proteins to procalcitonin. *Animal Health Research Reviews*, 23(1), 82-99.

II. **López-Martínez, M.J.,** Cerón J.J., Ortín-Bustillo A., Escribano D., Kuleš J., Beletić A., Rubić I., González-Sánchez J.C., Mrljak V., Martínez-Subiela S., Muñoz-Prieto A. (2022). A Proteomic Approach to Elucidate the Changes in Saliva and Serum Proteins of Pigs with Septic and Non-Septic Inflammation. *International Journal of Molecular Sciences*, 23(12),6738.

III. **López-Martínez, M.J.,** Beletić, A., Kuleš, J., Rešetar-Maslov, D., Rubić, I., Mrljak, V., Manzanilla, E.G., Goyena, E., Martínez-Subiela, S., Cerón J.J., Muñoz-Prieto, A. (2022). Revealing the Changes in Saliva and Serum Proteins of Pigs with Meningitis Caused by Streptococcus Suis: A Proteomic Approach. *International Journal of Molecular Sciences*, 23(22),13700.

IV. Rodrigues, M.; **López-Martinez, M.J.,** Ortin-Bustillo, A.; Cerón, J.J.; Martinez-Subiela, S.; Muñoz-Prieto, A.; Lamy, E. (2023). Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by *Escherichia coli*. *Proteomes*, 11, 14.

V. **López-Martínez, M.J.**, Escribano D., Contreras-Aguilar M.D., García-Martínez J.D., Martínez-Subiela S., Cerón J.J. (2020). Salivary D-dimer in pigs: Validation of an automated assay and changes after acute stress. *The Veterinary Journal*, 259-260, 105472.

VI. **López-Martínez, M.J.,** Escribano, D., Ortín-Bustillo, A., Franco-Martínez, L., González-Arostegui, L.G., Cerón, J.J., Rubio, C.P. (2022). Changes in Biomarkers of Redox Status in Saliva of Pigs after an Experimental Sepsis Induction. *Antioxidants*, 11(7),1380.

VII. Valros, A., **López-Martínez, M.J.,** Munsterhjelm, C., López-Arjona, M., Cerón J.J. (2022). Novel saliva biomarkers for stress and infection in pigs: Changes in oxytocin and procalcitonin in pigs with tail-biting lesions. *Research in Veterinary Science*. 153,49-56.

VIII. **López-Martínez, M.J.,** Martínez-Subiela, S., Cerón, J.J., Ortín-Bustillo, A., Ramis, G., López-Arjona, M., Martínez-Miró, S., Manzanilla, E.G., Eckersall, P.D., Tecles, F., Escribano, D., Muñoz-Prieto, A. (2023). Measurement of Calprotectin (S100A8/A9) in the Saliva of Pigs: Validation Data of A Commercially Available Automated Assay and Changes in Sepsis, Inflammation, and Stress. *Animals*, 13(7),1190.

IX. **López-Martínez, M.J.**, Escribano, D., Martínez-Miró, S., Ramis, G., Manzanilla, E.G., Tecles, F., Martínez-Subiela, S., Cerón, J.J. (2022). Measurement of procalcitonin in saliva of pigs: a pilot study. *BMC Veterinary Research*, 18(1),139.

In addition, it is considered appropriate to include three research results in the PhD Thesis Annex. These results are related to the work carried out during this Thesis and will be submitted in the recent future for their possible publications.

I. Changes in Salivary Biomarkers of Stress, Inflammation, Redox Status, and Muscle Damage due to Streptococcus suis Infection in Pigs.

II. Comparison of Different Assays for the Procalcitonin Measurements in Pigs

III. Validation on an Assay for the Measurement of Presepsin in the Saliva of Pigs

ABBREVIATIONS

- **AOPP**: Advanced Oxidation Protein Products
- **ADA**: Adenosine Deaminase
- **ALB**: Albumin
- **ALDOA**: Aldolase A
- **ALT**: Alanine aminotransferase
- **AOPP**: Advanced Oxidation Protein Products
- **APCS**: Serum amyloid P-component
- **APOE**: Apolipoprotein E
- **APOA1**: Apolipoprotein A-I
- **APPs**: Acute Phase Proteins
- **AST**: Aspartate aminotransferase
- **CALP**: Calprotectin (S100A8/A9)
- **CEEA**: Comité Ético de Experimentación Animal (Animal Experimentation Ethics Committee)
- **CK**: Creatine Kinase
- **CK**-MB: CK-myocardial band
- **CRP**: C-reactive protein
- **CUPRAC**: Cupric reducing antioxidant capacity
- **CVs**: Coefficients of Variation
- **DAMPs**: Damage-Associated Molecular Patterns
- **DSC2**: Desmocollin-2
- **GSN**: Actin-depolymerizing factor
- ***E. coli**: Escherichia coli*
- **FEU**: Fibrinogen Equivalent Units
- **FOX**: Ferrous oxidation xylenol orange
- **FRAS**: Ferric reducing ability of saliva
- **HBB**: Hemoglobin subunit beta
- **Hp**: Haptoglobin
- **HRG**: Histidine-rich glycoprotein
- **IL-1β**: Interleukin-1β
- **IL-6**: Interleukin-6
- **LCN**: Lipocalin
- **LDH**: Lactate dehydrogenase
- **LLOQ**: Lower Limit of Quantification

- **LOD**: Limit of Detection
- **LPS**: Lipopolysaccharide
- **MODS**: Multiple Organ Dysfunction Syndrome
- **OBP**: Odorant binding protein
- **OBP2B:** Lipocalin cytosolic FA-bd domain-containing protein
- **OXT:** Oxytocin
- **PAMPs**: Pathogen-Associated Molecular Patterns
- **PCA**: Principal Component Analysis
- **PIP**: Prolactin-inducible protein
- **PMN**: Polymorphonuclear Leukocytes
- **POX-Act**: Peroxide activity
- **PRAN**: Plan Nacional contra la Resistencia a los Antibióticos (National Action Plan on Antimicrobial Resistance)
- **PRRs**: Pattern-Recognition Receptors
- **PSE (sCD14)**: Presepsin (soluble CD14)
- **ROC curve**: Receiver Operating Characteristic curve
- $\boldsymbol{R^2}$: Coefficient of determination
- ***S. suis****: Streptococcus suis*
- **SAA**: Serum Amyloid A
- **sAA**: Salivary Alpha-amylase
- **sTREM-1**: Soluble Tumor Necrosis Factor Receptor
- **TMT**: Tandem Mass Tags
- **TNF-α**: Tumor Necrosis Factor α
- **VCL**: Metavinculin
- **WHO**: World Health Organization
- **ZnO**: Zinc Oxide

INTRODUCTION

Sepsis is a severe and life-threatening condition characterized by a systemic inflammatory response triggered by an infectious agent that can finally lead to organ dysfunction and even death. The morbidity and mortality rates get higher if sepsis is not promptly recognized and well-treated. Moreover, the improper characterization of the causal agent of sepsis can lead to a misuse of antibiotics that contributes to the development of antibiotic resistance (Riedel & Carroll, 2013; Taylor, 2015; Weiss et al., 2015; Smyth et al., 2016; Luppi, 2017). However, the diagnosis of sepsis is still a challenge: the clinical symptoms of sepsis can overlap with those of other non-infectious conditions, making it difficult to differentiate and confirm the presence of sepsis; and the gold standard method, blood culture, has limitations, including time delays in obtaining results and a high incidence of false negatives (Gotts & Matthay, 2016; Singer et al., 2016; Jereb et al., 2019).

To address these challenges, researchers and clinicians increasingly focus on identifying biomarkers, most related to inflammation, for the early detection and characterization of the causal agent of sepsis. In this context, biomarkers can provide valuable information about the presence and severity of the condition, as well as its underlying causal infectious agent. In human medicine, several biomarkers are routinely used to early detect bacterial infections and guide in antibiotic stewardship, like procalcitonin (Pierrakos & Vincent, 2010; Liu et al., 2016; Matur et al., 2017; Sager et al., 2017). In veterinary medicine, even though inflammatory and infectious diseases lead to many health problems and economic losses in farms, this field is poorly explored. By expanding our knowledge of inflammatory and sepsis biomarkers and improving their detection and measurement techniques, we can lead to earlier intervention and appropriate treatment, ultimately improving the pig's health outcomes and welfare and reducing the development of antibiotic resistance and economic losses in farms. The most convenient sample to measure those biomarkers would be saliva, as it is considered a non-invasive sample that ensures animal welfare and allows for serial sample collections, even on the same day, and by non-trained personnel (Cerón, 2019; Wolf et al., 2020).

This doctoral thesis aims to progress in the knowledge of biomarkers of inflammation that can be measured in saliva, which could be helpful in the diagnosis and monitoring of sepsis in pigs.

OBJECTIVES

The specific objectives of this PhD Thesis are to provide an advance in the knowledge of biomarkers of inflammation that could be potentially useful in the diagnosis and monitoring of sepsis in the saliva of pigs:

- **Objective 1.** Bibliographic research about the knowledge of sepsis and the current most common biomarkers used to diagnose and monitor this pathological state in veterinary medicine. This research resulted in the paper nº 1 (indicated in the *Articles* section).

- **Objective 2.** Identification of new potential biomarkers using proteomics techniques in:
    - An experimental model of septic and non-septic inflammation through administering LPS of *E. coli* and turpentine oil to pigs.
    - Samples from commercial farms with meningitis due to *S. suis*.
    - Samples from commercial farms with diarrhoea caused by *E. coli.*

  The results of this objective are published in the papers (indicated in the *Articles* section) nº 2 to 4.

- **Objective 3.** Validation and measurement of various biomarkers of inflammation, oxidative stress, welfare, or muscle damage, with potential application in septic inflammation and study of their possible changes in sepsis and other conditions. The results of this objective are published in the papers (indicated in the *Articles* section) nº 5 to 8 and are currently submitted for publication in Experiment 1 in the Annex.

- **Objective 4.** Development and validation of new assays to diagnose sepsis: procalcitonin and presepsin. The results of this objective are published in the paper (indicated in the *Article section)* nº 9 and will be submitted for possible publication in the future in two experiments described in the *Annex* section (Experiments 2 and 3).

EXTENDED SUMMARY

1. GENERAL REVIEW

1.1. Sepsis and SIRS

Sepsis occurs when an infectious agent triggers a systemic inflammatory response syndrome (SIRS) with an imbalance between proinflammatory and anti-inflammatory mechanisms. It has been described that more than 70% of cases of sepsis are caused by bacteria, leading generally to pneumonia and infections in different tissues (urinary tract, intra-abdominal or skin). However, sepsis can also be triggered by other infectious agents like viruses, fungi, or parasites, and the rate of this kind of cases is probably underestimated (Weiss et al., 2015).

Regardless of the causal agent, sepsis is considered one of the primary causes of severe illness in humans and domestic animals worldwide (Alberti et al., 2003; Taylor, 2015). It can lead to disturbances in hormonal, metabolic, cardiovascular, and coagulation systems, among others (Hotchkiss & Karl, 2003; Singer et al., 2016; Smyth et al., 2016). In addition, the dysregulated inflammatory response can finally lead to multiple organ dysfunction syndrome (MODS) and even death (Levy et al., 2003; Riedel & Carroll, 2013). For all the above reasons, it is critical to rapidly diagnose sepsis to start the most appropriate treatment as soon as possible.

1.2. The diagnosis of sepsis

The first step in diagnosing sepsis is usually based on the presence of two or more SIRS criteria, a set of clinical signs that can be used to identify the presence of a systemic inflammatory response in the body. The SIRS criteria consist of the evaluation of several variables: general (like changes in body temperature, heart or respiratory rate, altered mental status), inflammatory (leukocytosis/leukopenia, immature forms of white cells, increased inflammatory biomarkers), hemodynamic (hypotension, elevated cardiac index), organ-dysfunction (hypoxemia, coagulation abnormalities) and tissue-perfusion (hyperlactatemia, decreased capillary refill) (Angus & van der Poll, 2013; Spoto et al., 2020). However, as stated before, SIRS can occur due to aseptic causes; thus, the causal infectious agent should be detected to confirm the sepsis. However, the diagnostic of the causal agent is complex due to several factors: commonly, various infectious agents (viral, fungal, parasitic, and bacterial) are involved at the same time; there are aseptic pathologies that have similar symptoms to septic ones, and the initial clinical presentations may vary considerably based on individual factors. In addition, blood

culture, which is the current gold standard for detecting bacteremia, presents numerous limitations, such as a delay of 48 to 72 hours in obtaining results and a high incidence of false negative results (Gotts & Matthay, 2016; Singer et al., 2016; Jereb et al., 2019). In addition, these problems are exacerbated in farms, where the health vision is more collective than in humans, diseases can spread faster due to the intensive nature of livestock farming, and economic resources are more limited. All these factors make it challenging to diagnose and correctly treat the animals, which leads to high morbidity and mortality, a lot of economic losses and a high incidence of misuse of antibiotics (Luppi, 2017).

The misuse of antibiotics is one of the factors that contribute to the increase in antibiotic resistance, a problem with a significant impact on public health that was recognized by the World Health Organisation (WHO) in 2014. This problem is leading to growing restrictions on the use of these drugs in livestock farming, especially in swine production, in which the consumption of antibiotics is the highest among the livestock species in many countries (Murphy et al., 2017; Korsgaard et al., 2020). The restrictions in terms of antibiotic use in veterinary started with the ban of antibiotics as growth promoters in 2005 (Millet & Maertens, 2011), was followed by the ban of antibiotics in the feed (MEPs, 2016) and, recently, by the ban of medicinal products containing zinc oxide (ZnO), which is currently leading to a need to find alternatives (Bonetti et al., 2021).

Spain holds a prominent position in swine production, ranking second among European Union countries in exporting pork products (Bellini, 2021). Therefore, antibiotic consumption in the porcine industry in our country occupies a high position in the European Union. However, in the last years, Spain is showing a decrease in antibiotic usage in pig farming (European Medicines Agency, 2021), which can be mainly attributed to the implemented measures initiated by the European Union in 2014 and that in our country were translated into a National Action Plan on Antimicrobial Resistance (PRAN). In Spain, the PRAN is structured in six primary lines of action and includes the development of quick diagnostic laboratory methods that can help the veterinarian in the decision-making on the need for antibiotic therapy (PRAN, 2022). Biomarkers could help in the early detection and monitoring of inflammatory diseases and the characterization of their causal agent.

1.3. The picture in the porcine industry

Inflammatory and infectious diseases in swine are common problems at all stages of production. Most health problems in farms are related to respiratory and digestive problems caused by infectious agents, especially bacteria. In addition, the characteristics of the current intensive production of swine make easier the fast spread of these agents.

Regarding bacterial infections, there are two main types: the ones caused by primary agents, such as *Mycoplasma hyopneumoniae or Actinobacillus pleuropneumoniae;* and the opportunistic infections, such as the ones produced by the bacteria *Pasteurella multocida, Haemophilus parasuis or Actinobacillus suis*, which are produced secondary to viral infections or other host intrinsic factors (Brogden & Guthmiller, 2002). Nevertheless, this classification is not always that simple, as bacteria like *Streptococcus suis* or *Escherichia coli* usually coexist harmlessly with pigs, but with the pathogenic capability to induce diseases in specific situations, sometimes due to environmental or host factors, and other times due to the dose or virulence of the bacteria strain (Croxen et al., 2013; Gomes et al., 2016; Obradovic et al., 2021). All these factors make bacterial diseases difficult to diagnose, with very negative consequences for the pig industry.

1.4. Biomarkers of inflammation in sepsis

Using biomarkers for early sepsis detection, categorization, and monitoring can significantly enhance its management and treatment.

This field is in its early stages in veterinary, and much remains to be explored. There have been significant advances in the last years in expanding the range of possible biological samples to be used for measuring biomarkers beyond serum, such as saliva, hair, or faeces, which is crucial in the pig, a species in which blood extraction is highly stressful (Merlot et al., 2011; Martínez-Miró et al., 2016). One of the most convenient biological samples in pigs is saliva, a non-invasive method that guarantees swine welfare and an easy and fast sample to collect, even several times on the same day.

The biomarkers that have been most used in the last years in veterinary medicine to detect infectious and inflammatory diseases are acute phase proteins (APPs), such as C-reactive protein (CRP), haptoglobin (hp) or serum amyloid A (SAA), which have in general rises of high magnitudes and allow disease detection at early stages and even on subclinical cases, making them the most sensitive markers of inflammation. In addition, APPs can be measured easily in saliva samples (Cerón, 2019). Nevertheless, APPs have

several limitations, like the low specificity to detect the causal agent, as the increases can be similar in other inflammatory aseptic conditions, like immune-mediated pathologies. Still, these proteins can give valuable information but must be complemented with more specific biomarkers (Ercan et al., 2016).

Recently, a growing interest has been in broadening the spectrum of biomarkers to detect sepsis in animals. In human medicine, more than 170 potential biomarkers are described for assessing sepsis diagnosis and prognosis (Pierrakos & Vincent, 2010; Reinhart et al., 2012; Liu et al., 2016). Moreover, some of these biomarkers are routinely used in clinical practice in the sepsis diagnosis, prognosis, and guidance through antibiotic stewardship, like procalcitonin (PCT). PCT concentrations in physiologic conditions are very low, as almost all is converted into mature calcitonin. In septic conditions, PCT is massively produced and released directly into the blood, increasing its concentrations even up to thousands of folds (Becker et al., 2010; Matur et al., 2017; Sager et al., 2017). Other proteins such as calprotectin (CALP, S100A8/A9) or presepsin (PSE, sCD14) have also an increasing presence in daily human clinical practice. CALP is a calcium-binding heterodimer that belongs to the S100 protein family that is involved in several proinflammatory functions, and PSE is a protein very related to sepsis because it results from the cleavage of the receptor of the immune system CD14 that occurs after having contact with bacterial antigens. Nevertheless, there are hardly any suitable methods to measure them in veterinary medicine, and thus, little is known about how these specific sepsis biomarkers behave in animals.

Additionally, it is necessary to search for new potential biomarkers that could be more specific to different animal species or veterinary diseases. For this purpose, proteomic techniques are a powerful tool to identify protein profiles in animal biological fluids, a pivotal point in biological samples that are less well-understood due to a more recent development, such as saliva. Moreover, proteomics allows identifying protein profiles in different health states, thus making it possible to find biomarkers that may change in certain diseases (Ceciliani et al., 2014). Regarding sepsis, the study of salivary and serum proteomes through proteomics techniques can provide a better understanding of the pathogenesis and potentially discover a wide range of biomarkers that could help characterize it (Sharma & Salomao, 2017).

That is the starting point of this doctoral thesis, which has the aim of learning more about existing biomarkers of inflammation and infection, searching for new possible

biomarkers, and developing new analytical methods that allow us to measure them in pigs, focusing on the use of saliva as the biological sample.

2. GENERAL METHODS

2.1. Ethical considerations

All experimental animal procedures were conducted according to the Three Rs principle of Animal Experimentation following Spanish (RD53/2013) and European (Directive 2010/63/EU) legislation. The experiments that required animal handling were approved by Bioethical Committee from the University of Murcia (Comité Ético de Experimentación Animal, CEEA), under the protocol numbers CEEA 171/2015, 235/2018, and 563/2021. The procedures for monoclonal antibody production, in addition to being approved by their corresponding CEEA, followed the European regulations on monoclonal antibody production (ECVAM Workshop 23, 1997). The transport model of pigs that was used to evaluate several biomarkers was performed according to the recommendations described in Directive 2001/88/EC, 2001 and Directive 2001/93/EC, 2001. Additionally, the study protocol used in the article in collaboration with the Research Centre for Animal Welfare in Finland was considered ethically acceptable by the University of Helsinki Viikki Campus Research Ethics Committee (Statement 2/2022).

2.2. Polyclonal and monoclonal antibody production and purification

2.2.1. Polyclonal antibody production

Regarding the polyclonal antibody of PCT, it was performed according to standard protocols (University of California Berkley Animal Care and Use Committee, 2009) in a New Zealand rabbit (female, 2.5 kg, 3-months old) supplied by the commercial farm Granja San Bernardo (Navarra, Spain). The rabbit was immunized using 100 µg of porcine PCT (Biovendor, RD572451100) as an antigen, diluted in NaCl and emulsified in Freund's adjuvant (complete in the first immunization, incomplete in the booster ones) in a total of 0.2 ml subcutaneously (Leenaars & Hendriksen, 2005). A week after each immunization, blood was collected via the auricular vein of the rabbit.

Large volumes of antisera were needed for the polyclonal antibody of PSE; therefore, it was performed in goats. The protocol is very similar to rabbits, but the amount of the antigen that was injected subcutaneously was 500 µg. The antigen, which was a custom peptide with part of the porcine PSE sequence developed by a commercial company

(Proteogenix, France) to increase immunogenicity, was diluted in NaCl and emulsified in Freund's adjuvant, in this case the incomplete one in all the immunizations, in a total of 0.5 ml per immunization. After immunizations, blood collection was performed via the goat's jugular vein.

In both cases, the obtained sera were screened through ELISA and western blot to evaluate the antibody titration and affinity and frozen at -80 ºC until further purification.

2.2.2. Polyclonal antibody purification

The sera obtained in the previous section were thawed, filtered with a 0.45 μm commercial filter (Millipore, Massachusetts, USA) and purified with an automated liquid chromatography system (ÄKTA pure, GE Healthcare Life Sciences), passing the sera through a HiTrap protein G HP affinity column according to the manufacturer's instructions (GE Healthcare Life Sciences, Munich, Germany).

2.2.3. Monoclonal antibody production

To produce the monoclonal antibodies, three BALB/c mice were immunized with 50 μg of the antigen diluted in NaCl and 200 μl of Freund's adjuvant, complete in the first immunization and incomplete in the booster ones. The antigens used were commercial PCT (Biovendor, RD572451100) and the custom peptide with part of the porcine PSE sequence mentioned in the polyclonal antibody production section, respectively. The serum of the mice was collected via the retro-orbital sinus and tested in ELISA to assess the immune response against the selected antigens and select the mouse with the highest affinity. Then, the chosen mice were euthanized by cervical dislocation, and the B lymphocytes of their spleen were seeded in 96-well plates and fused with myeloma cells to produce hybridomas, following the protocol of Yokoyama (1999). Finally, the supernatants were tested in ELISA, and the clones from the wells considered positive to the target antigens were selected and cloned by serial dilutions to get clones producing the same type of monoclonal antibody in each case. Finally, these clones were seeded in 75-cm^2 flasks for antibody production.

2.2.4. Monoclonal antibody purification

The supernatants from the cell culture of hybridomas obtained in the previous section were collected in 75-cm^2 flasks each week and stored at -80 ºC until the total aimed

volume was obtained. Then, the supernatants were thawed, precipitated with ammonium sulfate (9.7 g per 25 mL of supernatant, 30 min under agitation, centrifugation at 4000× *g* for 30 min, removal of the supernatant, and precipitate reconstitution with PBS) and concentrated with 30 kDa MWCO ultracel regenerated cellulose devices (Merck, Darmstadt, Germany) before antibody purification. After that, monoclonal antibodies were purified with a HiTrap Protein G HP column (GE Healthcare Life Sciences, Munich, Germany) using a chromatography system (ÄKTA pure, GE Healthcare Life Sciences).

2.3. Sampling procedures

Saliva in pigs was collected with polypropylene sponges (Esponja Marina, La Griega E. Koronis, Madrid, Spain) cut into approximately 5 x 2 x 2 cm pieces and clipped to flexible metal rods or forceps. The sponges were gently introduced into the pig's mouth unless the pigs voluntarily came to chew the sponges. Once thoroughly moistened, the sponges were placed into Salivette® tubes (Sarstedt, Aktiengesellschaft and Co. Nümbrecht, Germany).

Blood samples in pigs were obtained by puncturing the jugular vein and collected into vacuum plain tubes (BD Vacutainer, Franklin Lakes, NJ, USA).

All samples were kept at 4–8 °C in a portable refrigerator until arrival at the laboratory, where the vacutainer or the Salivette tubes were centrifuged at 3000× *g* and 4 °C for 10 min to obtain serum and saliva supernatant, respectively. Then, the samples were transferred into Eppendorf tubes and stored at −80 °C until analysis.

2.3.1. Septic and non-septic inflammation induction in pigs

An experimental septic and non-septic model of inflammation to evaluate different biomarkers was performed in this PhD thesis. The inductions were performed in 15 male pigs in the mid-fattening period, 5 per group. In the first group (*n*=5; control group), a NaCl injection (2 mL) was administered by intramuscular route. The second group (*n*=5; LPS group) received a unique dose of 30 μg/kg LPS from *Escherichia coli* (LPS; O55:B5, Sigma-Aldrich, previously reconstituted with sterile saline solution) by intramuscular injection (Wyns et al., 2015; Petry et al., 2017). In the third group (*n*=5, TURP group), 8 mL of TURP (oil of turpentine purified, Sigma–Aldrich) were administered through two 4 mL subcutaneous injections in each front flank per animal. All administrations were completed between 8 and 9 a.m. Saliva and paired blood samples were obtained 24 h

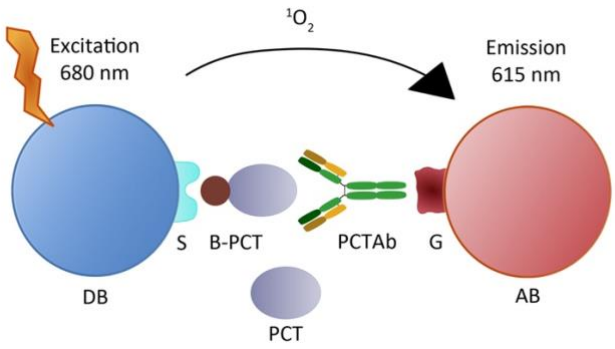
before (basal time) these administrations and 3, 6, 24, and 48 h after them. However, not all the samples were used for all the studies due to insufficient sample quantity. Basal, 24 h, and 48 h samples were obtained at 8 a.m.

2.4. Assays development

The AlphaLISA (amplified luminescent proximity homogeneous assays) technology (PerkinElmer, Inc., Massachusetts, USA) was the format used to develop immunoassays in this PhD thesis. This technology is based on luminescent oxygen-channelling chemistry through two types of beads: acceptor and donor. The main principle of this type of assay is that laser irradiation is produced at 680 nm; if binding of the molecules captured on the beads has occurred, it triggers a release of oxygen molecules resulting in intense luminescent emission at 615 nm. AlphaLISA immunoassays can be designed in a sandwich or competitive configuration and have advantages, such as the potential use of minimal sample quantities (the total volume per well is usually 50 µl) or no plate washing required.

The developed assay to measure procalcitonin was an indirect competitive assay with a polyclonal antibody. The schematic diagram of how the method works is represented in Fig. 1.

Fig. 1. *Schematic picture of the AlphaLISA reaction for procalcitonin detection. DB, donor bead; S, streptavidin; B-PCT, biotinylated procalcitonin; PCT, procalcitonin of the sample; PCTAb, Anti-procalcitonin polyclonal antibody; G, protein G; AB, acceptor bead.*



For optimizing assay conditions, different concentrations of all components were evaluated. The performance of each combination was tested with a constant amount of standard with known concentration, and assay buffer was used as a blank. Then, the magnitude of signal change (expressed as counts in AlphaLISA assays) and the maximum

obtained signal and the buffer/protein ratio were evaluated in each condition, which includes the recommended standard concentration ranges: acceptor beads coated to protein G (20-40 μg/mL), antibody (0-10 nM), biotinylated protein (0-20 nM) and donor beads (20-40 μg/mL). The protein was labelled with the commercial biotin solution EZ-LinkTM Micro Sulfo-NHS-Biotin, No-WeightTM Format (Thermo Scientific, USA). In addition, samples with high and low analyte concentrations were diluted from 1:2 to 1:16 to assess which dilution showed the best linearity. The performance of different buffers (PBS, alpha buffer and universal buffer, the last ones from PerkinElmer, Inc., Massachusetts, USA) were tested with the standard and several samples. As a standard, a commercial porcine PCT (Biovendor R&D, Brno, Czech Republic) was used, and the curve was prepared with concentrations ranging from 10 to 10000 ng. Results were expressed in μg/L.

2.5. Analytical validation of the assays

Several assays of this PhD thesis had to be analytically validated to evaluate the methods' precision, accuracy, and sensitivity in pig samples. These proceedings were performed according to previous protocols (Andreasson et al., 2015; Tecles et al., 2007), and the parameters evaluated were:

- Imprecision. It was assessed by analyzing five replicates of each sample with low, medium, and high concentrations of the analyte, simultaneously in the case of intra-assay, and on different days (and different aliquots, to avoid freeze and thawed cycles) in inter-assay. Intra and inter-assay variations were calculated employing coefficients of variation (CVs) in percentage. In all cases, CVs lower than 20% were considered acceptable.
- Accuracy. It was evaluated through linearity in serially diluted samples, comparing the measured analyte concentrations and the expected analyte concentration through regression analyses; and spike recovery experiments, adding different amounts of a known concentration of the analyte to samples and calculating the CVs (%) comparing the expected versus the observed values.
- Sensitivity. It was tested by determining the limit of detection (LOD) and lower limit of quantification (LLOQ). LOD is defined as the lowest concentration of the analyte that can be distinguished from the zero value and is calculated with the mean value of 12 replicates of assay buffer plus two standard deviations. LLOQ

is the lowest amount of the analyte that can be measured with acceptable precision and is determined by measuring a serially diluted sample repeating each dilution five times within the same run assay and calculating the CVs (%).

2.6. Proteomic analysis

2.6.1. Gel proteomics

2.6.1.1. Sample preparation

In all cases, the total protein concentration of the samples was determined through the BCA assay (Thermo Scientific, Rockford, IL, USA). For the SDS-page, individual samples with 9 μg of protein were used; for the 2DE technique, pools of pig saliva samples were prepared with a total protein of 275 μg to run them in duplicate. Each individual sample and pool was lyophilized and stored at −28 ºC.

2.6.1.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page)

Proteins were separated by SDS-PAGE gel electrophoresis on 12% acrylamide gels. Each lyophilized saliva sample was reconstituted with 40 μL of sample buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% DTT and bromophenol blue) and heated for 5 min at 98 ºC to denature proteins. The electrophoresis was run at a constant voltage of 150 V. The gels were fixed in 40% methanol and 10% acetic acid, stained with Coomassie Brilliant Blue R-250, and distained with 10% acetic acid. Finally, LabScan software was used to acquire scanned images of the gels, and ImageLab software (Bio-Rad, Alges, Portugal) was used for gel analysis.

2.6.1.3. Two-Dimensional Gel Electrophoresis (2-DE)

Each lyophilized pool was reconstituted with 250 μL of solubilization buffer [7 M urea, 2 M thiourea, 4% (*w*/*v*) 3-(3-cholamidopropyl) dimethylammonium propane sulfonate (CHAPS), 2% (*v*/*v*) ampholyte mixture (IPG buffer pH 3-11, GE Healthcare, Chicago, IL, USA), and 40 mM dithiothreitol (DTT)]. The mixture was incubated and centrifuged to obtain the supernatants of the samples. Focusing was performed in a Multiphor II (GE, Healthcare, Chicago, IL, USA) at 12 ºC. After that, strips were equilibrated and ran in a 12% acrylamide gel through SDS-PAGE at 150 V. Staining was made with CBB-R250. The image acquisition of the gels was made by a gel scanner (ImageScanner III, GE Healthcare, Chicago, IL, USA) and Lab scan software (GE

Healthcare, Chicago, IL, USA), and the analysis was performed using the SameSpots software (v5.1.012, TotalLab, Gosforth, UK).

2.6.2. Gel-free proteomics

2.6.2.1. Sample preparation

The protein identification of the samples processed on SDS and 2DE gels was performed through HPLC-MS/MS Analysis. To this end, the bands and spots that differed between groups in relative amounts in both gels were selected and processed by digestion with trypsin following previous protocols before MS analysis.

In other studies of this PhD thesis, proteomic analysis has been performed exclusively with gel-free proteomics using the TMT-based quantitative approach after the LC-MS/MS Analysis. The saliva samples were centrifuged, precipitated overnight in the ice-cold acetone, and resuspended in 1% SDS in 0.1 M triethyl ammonium bicarbonate, and protein concentration was determined using the BCA assay. The following preparation steps were the same for the serum and saliva samples. 35 µg from the samples and internal standards were reduced with dithiothreitol (DTT), alkylated with iodoacetamide, and precipitated overnight with ice-cold acetone. After centrifugation, the protein pellets were dissolved in 0.1 M TEAB and digested with 1 mg/mL trypsin, and 19 µL of the specific TMT label was added to each sample for 1 hour until the addition of 5% hydroxylamine to quench the reaction. Five TMT-modified samples were randomly combined with the internal standard, aliquoted and dried before the LC-MS/MS analysis.

2.6.2.2. High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS/MS) Analysis

An HPLC/MS system consisting of an Agilent 1290 Infinity II Series HPLC connected to an Agilent 6550 Q-TOF mass spectrometer was used. Dry samples from trypsin digestion were resuspended in a buffer with water/acetonitrile/formic acid and injected into an Agilent AdvanceBio Peptide Mapping HPLC column, thermostated at 50 ºC, at a flow rate of 0.4 mL/min.

The data processing and protein identification was made on Spectrum Mill MS Proteomics Workbench (Rev B.06.00.201, Agilent Technologies, Santa Clara, CA, USA).

2.6.2.3. Liquid chromatography-tandem Mass spectrometry (LC-MS/MS) Analysis

A platform consisting of the Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) and the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used. The labelled peptides were dissolved, loaded onto the trap column (C18 PepMap100, 5 μm, 100A, 300 μm × 5 mm) and separated on the analytical column (PepMapTM RSLC C18, 50 cm × 75 μm). Two mobile phases were used to achieve the separation gradient. For ionization, the nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) with the 10 μm inner diameter SilicaTip emitter (New Objective, USA) was used.

For protein identification and quantification, the SEQUEST algorithm with the Proteome Discoverer software (version 2.3., ThermoFisher Scientific) was used, searching against Sus scrofa FASTA files (downloaded from Uniprot database on December 2, 2020, 150,392 sequences). The false discovery rate (FDR) was set at 5%, as calculated with the Percolator algorithm in the Proteome Discoverer workflow.

2.7. Biochemical analysis

Regarding the biomarkers studied in this PhD Thesis to assess inflammation, stress, redox status or muscle damage, several methods have been employed based on different techniques (Table 1):

- Automated commercial kits in Olympus AU400 (Beckman Coulter): D-dimer, sAA, ADA and isoenzymes ADA1 and ADA2, ALDOA, CALP, CK, CK-MB, lactate, LDH, AST, ALT, total proteins, CUPRAC, FRAS, TEAC, uric acid, AOPP, FOX, POX-Act and d-ROMS.
- Automated chemiluminescent immunoassays in Immulite 1000 (Siemens Healthcare Diagnostic): troponin I and cortisol.
- ELISA kits: calgranulin c.
- AlphaLISA assays: cortisol, PCT, Hp and oxytocin.

Table 1. *Biochemical parameters and methods employed in this PhD thesis. Each method is stated if it was validated for its use in pig saliva before the experiments of this PhD thesis. The methods that were not previously validated in pig saliva were validated in the studies included in this thesis. *These assays were performed using the Olympus AU400 (AU400 Automatic Chemistry Analyser, Olympus Europe GmbH, Hamburg, Germany) following the manufacturer's recommendations.*

Biomarker	Method employed	Previous validation
D-dimer	Automated commercial kit (Diazyme Laboratories, California, USA).*	No
Salivary Alpha-amylase activity (sAA)	Automated commercial kit (Beckman Coulter, California, USA).*	Yes (Fuentes et al., 2011)
Adenosine Deaminase (ADA) and its isoenzymes ADA1 and ADA2	Automated commercial kit (Diazyme Laboratories, California, USA). The specific ADA1 inhibitor EHNA (Merck KGaA, Darmstadt, Germany) was used for isoenzyme determinations. The isoenzyme ADA1 is calculated from the difference between both measurements.*	Yes (Tecles et al., 2018; Contreras-Aguilar et al., 2020)
Aldolase (ALDOA)	Automated commercial kit Randox Laboratories Ltd., Crumlin, UK).*	No
Calprotectin (CALP) (S100A8/A9)	Automated commercial kit (BÜHLMANN, Laboratories AG, Switzerland)* with two modifications: a solution of purified human CALP was used as Calibrator A to initially measure in saliva samples of pigs, being the units assessed in mg/L instead of µg/g; and then, Calibrator B was performed with a pooled saliva sample with a known concentration of CALP, in order to reduce the possible matrix effect.	No
Calgranulin C (S100A12)	ELISA commercial kit (Cloud-Clone Corp, Wuhan, China)	No
Creatine kinase (CK)	Automated commercial kit (Beckman Coulter, California, USA)*	No
CK-myocardial band (CK-MB)	Automated commercial kit (Beckman Coulter, California, USA)*	No
Lactate	Automated commercial kit (Beckman Coulter, California, USA)*	No
Lactate dehydrogenase (LDH)	Automated commercial kit (Biosystem, Barcelona, Spain)	Yes (Escribano et al., 2019)
Aspartate aminotransferase (AST)	Automated commercial kit (Beckman Coulter, California, USA)*	No

Alanine aminotransferase (ALT)	Automated commercial kit (Beckman Coulter, California, USA)*	No
Total protein concentration	Automated commercial kit (protein in urine and CSF, Spinreact, Spain)*	Yes (Escribano et al., 2015)
Troponin I	Automated chemiluminescent immunoassay with Immulite 1000, Siemens Healthcare Diagnostic	No
Cortisol	Automated chemiluminescent immunoassay with Immulite 1000, Siemens Healthcare Diagnostic	Yes (Escribano et al., 2012)
	In-house indirect competitive AlphaLISA (monoclonal antibody)	Yes (López-Arjona et al., 2020c)
Procalcitonin (PCT)	In-house indirect competitive AlphaLISA (polyclonal antibody)	No
Oxytocin (OXT)	In-house direct competitive AlphaLISA (monoclonal antibody)	Yes (López-Arjona et al., 2020b)
	In-house indirect competitive AlphaLISA (polyclonal antibody)	Yes (López-Arjona et al., 2021)
Haptoglobin (Hp)	In-house direct sandwich AlphaLISA (monoclonal antibody)	Yes (Contreras-Aguilar et al., 2021)
Cupric reducing antioxidant capacity (CUPRAC)	Automated in-house assay based on generating a complex containing Cu^{2+} and one chelating agent and its reduction to Cu^{1+} by the non-enzymatic antioxidants present in a sample.*	Yes (Rubio et al., 2019)
Ferric reducing ability of saliva (FRAS)	Automated in-house assay based on a reaction mixture containing ferric-tripyridyltriazine (Fe^{3+}) that is reduced to the ferrous (Fe^{2+}) form by the non-enzymatic antioxidants provided by the sample.*	Yes (Rubio et al., 2019)
Trolox equivalent antioxidant capacity (TEAC)	Automated in-house assay based on the enzymatic generation of ABTS (2,2′-azinobis [3-ethylbenzthiazoline-6-sulfonic acid]) radical.*	Yes (Rubio et al., 2019)
Uric acid	Automated commercial kit (Beckman coulter Inc, California, USA).*	Yes (Rubio et al., 2019)
Advanced oxidation protein products (AOPP)	Automated in-house previously described method (Witko-Sarsat et al., 1996) calibrated with Chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide in acidic conditions.*	Yes (Rubio et al., 2019)

Ferrous oxidation-xylenol orange (FOX)	Automated in-house method based on the oxidation of ferrous to ferric ions by lipid hydroperoxides in the sample as previously published (Arab & Steghens, 2004).*	No
Peroxide activity (POX-Act)	Automated in-house method based on a previously published assay (Tatzber et al., 2003) in which the oxidation of 3,5,3′5′-Tetramethylbenzidine (TMB) by peroxides in the sample is monitored.*	No
Salivary reactive oxygen-derived compounds (d-ROMS)	Automated in-house method based on monitoring the N,N-Diethyl-p-phenylenediamine radical cation concentration as previously described (Cesarone et al., 1999).*	No

3. EXPERIMENTAL DESIGN, RESULTS, AND DISCUSSION OF THE DIFFERENT OBJECTIVES

The following data will be presented according to the papers published in this PhD Thesis.

3.1. Objective 1

Objective 1 was covered by one study corresponding to article nº 1, in which bibliographic research was performed to deepen the knowledge of sepsis and the current most common biomarkers used to diagnose and monitor this inflammatory state in veterinary medicine.

3.1.1. Biomarkers of sepsis in pigs, horses and cattle: from acute phase proteins to procalcitonin (Article 1)

Sepsis is a complex inflammatory syndrome triggered by an infection that has severe consequences in human and veterinary health and economic aspects, such as increased morbidity, mortality, and misuse of antibiotics, especially when treatment is not started early. That is a huge problem in farm animals because antibiotics have been traditionally given on a massive scale, but now they are increasingly restricted.

The origin of sepsis relies on the interaction between the causal agent and the ‘pattern-recognition receptors’ (PRRs) present on cells of the innate immune response

(Fig. 2). These receptors can detect conserved structures of pathogens, the 'pathogen-associated molecular patterns' (PAMPs), but also other molecules produced by the host after damage, independent of the causal agent, known as 'damage-associated molecular patterns'. (DAMPs). That explains the similarities between sepsis and non-septic SIRS and the difficulty of diagnosing sepsis (Lewis et al., 2012; Faix, 2013).

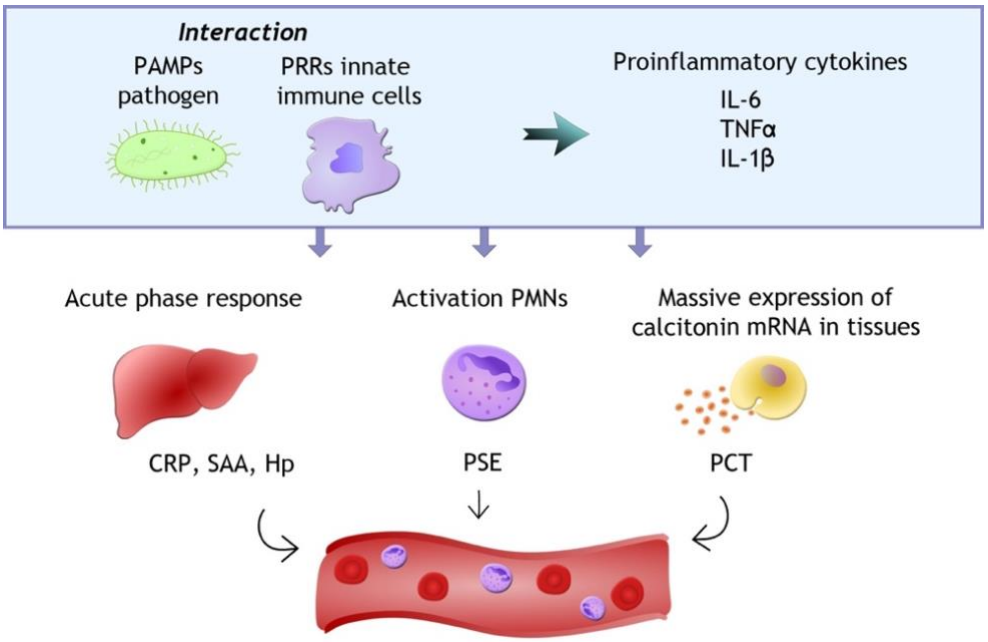
The interactions between the pathogen and cells trigger the production of proinflammatory cytokines, mainly interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β); also, the initiation of the innate immune response, inducing the production of acute phase proteins (APPs) in the liver, such as C-reactive protein (CRP), serum amyloid A (SAA), or haptoglobin (Hp). These interactions pathogen-cells also induce the activation of endothelial cells and production and attraction of polymorphonuclear leukocytes (PMNs) to the site of damage and into the general circulation. The latter leads to the release of molecules such as sCD14 (known as presepsin, PSE) or soluble tumor necrosis factor receptor (sTREM-1) from these receptors. In addition, both the direct stimulation of the pathogens themselves and the indirect stimulation by the cytokines induce the expression of calcitonin mRNA in numerous extrathyroidal tissues, leading to widespread production of the protein procalcitonin (PCT) (Cray, 2012; Faix, 2013; Nakamura et al., 2013).

Most of the mentioned molecules have been considered potential sepsis biomarkers in human medicine. Regarding animals, proinflammatory cytokines have been studied but are usually limited to research due to their short half-life in the blood. In clinical practice, the most frequent biomarkers used to detect sepsis are APPs such as CRP, sAA and Hp, which can provide an early diagnosis of inflammation even in subclinical cases. However, these proteins have low specificity in characterizing the causal agent of sepsis. In human medicine, the most promising biomarkers to detect the bacterial origin of sepsis are currently PCT and PSE, and there is a vast other range of other biomarkers under study. However, more information is needed on the application of these biomarkers in veterinary medicine.

This review has described the general concepts of sepsis and the current knowledge about three main groups of potential biomarkers of sepsis in pigs, horses, and cattle: APPs and cytokines, which have been traditionally used to assess inflammation; PCT, PSE, and other more recent proteins that are more specific sepsis markers; and other markers that can guide in sepsis monitoring.

Overall, each biomarker of the three groups could have a particular use in sepsis and provide complementary information. APPs and cytokines are not specific for sepsis but can rapidly detect the inflammation associated, monitor the evolution, and establish a prognosis. PCT, PSE, and other molecules, such as sTREM-1, LBP, and MMP-9, have enormous potential because they can improve the diagnosis of bacterial sepsis. Finally, the biomarkers of the third group provide additional information for prognosis and monitoring through the assessment of endothelial damage, organic dysfunction, and alterations in the coagulation system. Further studies should better define the ability and applications of these biomarkers, especially those with the potential to detect bacterial sepsis, and establish accurate cut-off values in veterinary medicine.

Fig. 2. *Proinflammatory response during sepsis. PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; IL-6, interleukin-6; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β; CRP, C-reactive protein; SAA, serum amyloid A; PMN, polymorphonuclear leukocytes; PSE, presepsin; PCT, procalcitonin. The authors created the figure and its images.*



3.2. Objective 2

Objective 2 was covered by three published studies corresponding to the articles nº 2 to 4. The main objective of these articles was the identification of new potential biomarkers. For this purpose, proteomics techniques were used in:

- An experimental model of septic and non-septic inflammation through administering LPS of *E. coli* and turpentine oil to pigs.

- Samples from pigs of commercial farms with meningitis due to *S. suis*.
- Samples from pigs of commercial farms with diarrhoea caused by *E. coli.*

3.2.1. A proteomic approach to elucidate the changes in saliva and serum proteins of pigs with septic and non-septic inflammation (Article 2)

- Aims and experimental design

This study aimed to evaluate the potential changes in the proteome of saliva and serum of pigs with LPS-induced sepsis, which could help better understand the pathophysiological mechanisms involved in the process of sepsis and discover potential biomarkers that can improve its diagnosis and monitoring. Proteomic techniques allow the study of a complete protein profile of a sample, evidencing the alterations associated with specific metabolic pathways (Bilić et al., 2018). Gel-based proteomics has already been used to investigate swine serum proteome after LPS administration (Olumee-Shabon et al., 2020). Nevertheless, the gel-free mass spectrometry-based proteomics provides higher quantification accuracy than the gel-based techniques (Abdallah et al., 2012), especially with the use of isobaric tagging through tandem mass tags (TMT), which increases the sensitivity of the analysis (Baeumlisberger et al., 2010). In addition, the salivary proteome could show differences in the number and types of proteins that change under particular conditions compared to serum, providing complementary information, as it has been seen in other diseases and species (Franco-Martínez et al., 2020; Muñoz-Prieto et al., 2022).

The proteomic analysis in septic and non-septic inflammatory conditions was performed in growing pigs from the previously reported LPS and turpentine oil administration model. Saliva and blood samples used in this experiment were collected 24 h before the experiment (basal), and 6 (T6) and 24 h (T24) post-administrations.

After the proteomic analysis, it was also performed the validation of aldolase A (ALDOA), one of the proteins that showed higher changes in LPS-induced pigs in the proteomic study. To this end, the first step was the complete analytical validation of a commercially available automated reagent kit to measure ALDOA activity in pig saliva. Then, saliva samples from the experimental model of LPS and turpentine were measured. Also, saliva samples from two groups of Large White weaning pigs were collected: one group consisted of pigs diagnosed with a spontaneous meningitis outbreak due to *S. suis* (n=11) in a commercial farm, and the other of clinically healthy pigs (n=13). The animals

with meningitis had clinical signs compatible with this disease (ataxia, anorexia, lateral recumbency, and padding) (Obradovic et al., 2021) and were positive for the presence of *S. suis* in blood culture.

- Results and discussion

In our experimental conditions, the saliva and serum proteome of pigs showed changes in septic and non-septic inflammation. However, a higher number of proteins changed in abundance in the saliva of the septic group (18 vs 9 proteins).

- The main changes found in saliva were:

- In LPS-induced pigs, ALDOA and SERPINB12 showed the highest increases, and no significant differences were demonstrated in the non-septic model. GO analysis in saliva showed that 11 different GO terms were significantly associated with the LPS-treated pigs, such as the organonitrogen compound metabolic process, tissue development, regulation of the developmental process, and lipid transport (Fig. 14).
    - ALDOA is a glycolytic enzyme whose family has a close relationship with muscle damage, the development of the brain, and ATP production. In the muscles of rats treated with LPS, it has been observed an increase in ALDOA expression. In addition, it acts as an adhesin in the membrane surface of different pathogens, being a potent stimulator of immune response in humans (Wu et al., 2008; Tunio et al., 2010; Yan et al., 2011; Goldman et al., 2016).
    - SERPINB12 is an inhibitor of trypsin-like serine proteinases that is present in epithelia and tissues and could be related to the defense and compensatory effects of the organism against bacterial infection (Askew et al., 2001; Niehaus et al., 2015).

- In the non-septic model, two specific proteins, albumin (ALB) and histone H4, were higher only in this group. In the GO analysis, changes in humoral immune response and serine-type endopeptidase activity were observed (Fig. 15).
    - The presence of albumin in porcine saliva could be more related to the local production than to the ultrafiltration of serum since the increased levels of albumin were not found in the serum. Further studies are

necessary to clarify why the increase in salivary albumin was found only in the non-septic inflammation group.

- Regarding histone H4, the family of these essential proteins are located in the cell's nucleus and play proinflammatory functions when released into the extracellular environment. In previous studies, elevated concentrations were observed in sepsis (Ekaney et al., 2014), but in our study that rise has only occurred in the non-septic model, which requires further research.

Fig. 14. *Significantly enriched GO terms among differentially expressed proteins in the saliva of pigs with sepsis. Edges link proteins (diamonds) to their associated GO terms (circles). Proteins are colored in green if overexpressed or in red if down-expressed. GO terms are colored accordingly to the proportion of over-/down-expressed proteins. GO term shape and font size are proportional to GO term significance, but all included ones showed a p-value<0.05.*

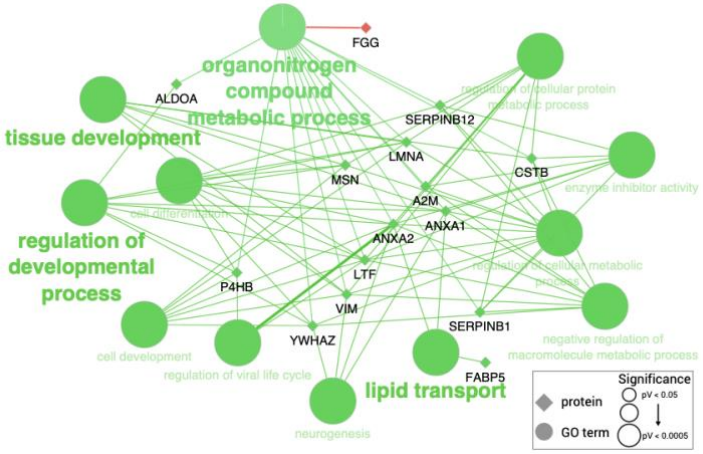
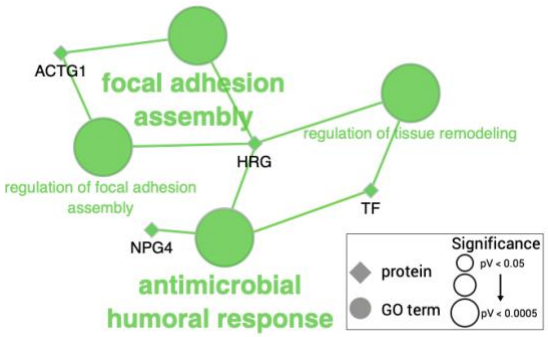


Fig. 15. *Significantly enriched GO terms among differentially expressed proteins in the saliva of pigs with non-septic inflammation. Edges link proteins (diamonds) to their associated GO terms (circles). Proteins colored in green are overexpressed. GO terms are colored accordingly to the proportion of over-/down-expressed proteins. GO term shape and font size are proportional to GO term significance, but all included ones showed a p value<0.05.*



- In serum, increases in APPs were detected in both conditions:

  - The LPS-induced pigs showed differences in the abundances of 30 proteins. The most outstanding increase was observed in apolipoprotein (APOE), which showed significant changes in septic but not in non-septic inflammation. This protein has been related previously to the risk of sepsis in human medicine (Shao et al., 2020). GO analysis enriched 34 terms among the altered serum proteins, generally related to the regulation of the response to external stimulus and apoptotic processes, and the negative regulation of blood coagulation. In the case of upregulated proteins, they were also associated with other processes like establishing localization to the extracellular region and regulating protein secretion.

  - In the turpentine group, a change in the relative abundance was observed for 26 proteins, being the most upregulated CRP, two SERPIN domain-containing proteins, Hp and lipopolysaccharide-binding protein (LBP), showing their higher expressions 24 h after the treatment. GO enrichment analysis stated that, in general, the proteins were related to hormone transport and response to chemicals. The upregulated proteins were more associated with vasoconstriction, protein polymerization and the positive regulation of peptide hormone secretion.

Regarding the analytical validation of the automated assay to measure ALDOA, it showed an imprecision <10% and a high linearity, with r^2>0.99. The lower quantification (LLOQ) and detection (LOD) limits were set at 1.3 and 0.1 U/L, respectively.

The activity of salivary ALDOA was significantly higher in the LPS-induced group at T6h compared with basal values (p=0.020). In contrast, no significant differences in the ALDOA activity were observed at T24h (Fig. 16a). Regarding the turpentine-induced group, ALDOA activity showed no significant differences in comparison with the basal values at 6 h and at 24 h (Fig. 16b).

In addition, pigs with meningitis caused by *S. suis* showed significantly higher activity levels of salivary ALDOA than healthy controls (p=0.001, Fig. 17).

Fig. 16. *Salivary ALDOA activity levels (U/L) in LPS-induced pigs (A) and turpentine-challenged pigs (B). Basal: 24 h before LPS or turpentine injection; T6 and T24: 6 or 24 h after LPS or turpentine-injection.*

*The lines indicate the minimum, median, and maximum values. Asterisks indicate statistically significant differences (*p<0.05) with basal time. Circles represent the sample values.*

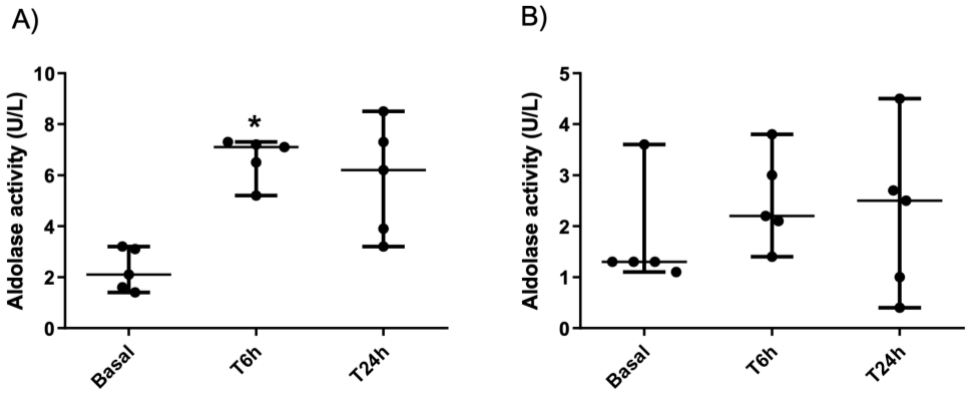
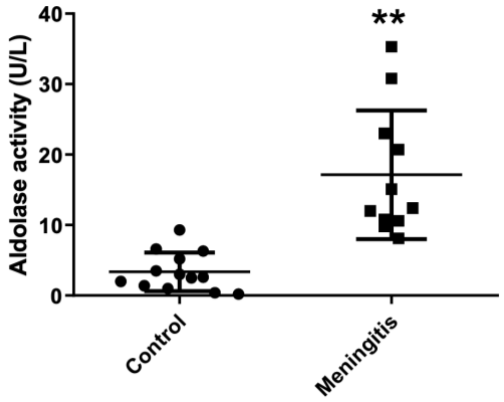


Fig. 17. *Salivary ALDOA activity (U/L) in pigs with meningitis compared with healthy controls. Lines indicate the minimum, median, and maximum values. Asterisks indicate statistically significant differences (**p=0.001). Circles and squares represent the sample values of control and meningitis groups, respectively.*



These results indicate that proteins in saliva and serum can change differently in septic and non-septic inflammation, reflecting different pathophysiological mechanisms. Therefore, saliva could provide complementary information to serum, and some of the proteins that changed could be biomarkers in septic and non-septic inflammation.

3.2.2. Revealing the Changes in Saliva and Serum Proteins of Pigs with Meningitis Caused by Streptococcus Suis: A Proteomic Approach (Article 3)

- Aims and experimental design

This study aimed to investigate the possible changes in the salivary and serum proteome profile of piglets with meningitis due to *S. suis*, a Gram-positive bacteria with

an increasing zoonotic potential worldwide that can cause meningitis, arthritis, pneumonia, or endocarditis, leading to a high mortality and morbidity in pigs. Saliva proteome analysis would help clarify pathophysiological mechanisms and looking for new biomarkers to diagnose and track *S. suis* infection.

The LC-MS/MS TMT proteomic approach was used to analyze saliva and serum samples. The animals used were 20 male Large White weaning pigs from two groups: the control group with clinically healthy pigs (HP, *n*=10) and the disease group, consisting of pigs diagnosed with meningitis due to *S. suis* (MP, *n*=10). All animals in the meningitis group presented compatible clinical symptomatology (ataxia, anorexia, lateral recumbency, and padding) (Borges-Rodriguez et al., 2021; Obradovic et al., 2021) and were *S. suis* positive in blood culture and PCR.

For the validation of proteomic results through the measurement of ADA, additional animals were included, consisting of 19 more with *S. suis*-associated meningitis, and 19 healthy pigs of the same age, all sampled by the same approach that was used for the proteomic study.

- Results and discussion

In saliva, a total of 21 proteins showed different relative abundances between the control and diseased groups (Fig. 18), whose main molecular functions are mainly related to the binding capacity and catalytic activity.

- 11 proteins had higher relative abundance in meningitis, highlighting:
    - VCL, a cytoskeletal protein present among other tissues in the cardiac muscle that could be potentially associated with muscle damage and related to the seizures usually presented in meningitis. Similarly, another upregulated protein in our study was the actin-depolymerizing factor (GSN), whose family is involved in muscle contraction (Belkin et al., 1988; Lee et al., 2019; G. Yin et al., 2022).
    - DSC2, a cadherin present in desmosomes and involved in the calcium-dependent mechanism for cell-cell adhesion, could suggest myocardial damage associated with the *S. suis* infection. This cadherin has been previously related to cardiac alterations such as myocardial inflammation and fibrotic remodelling in mice or rhythm problems in

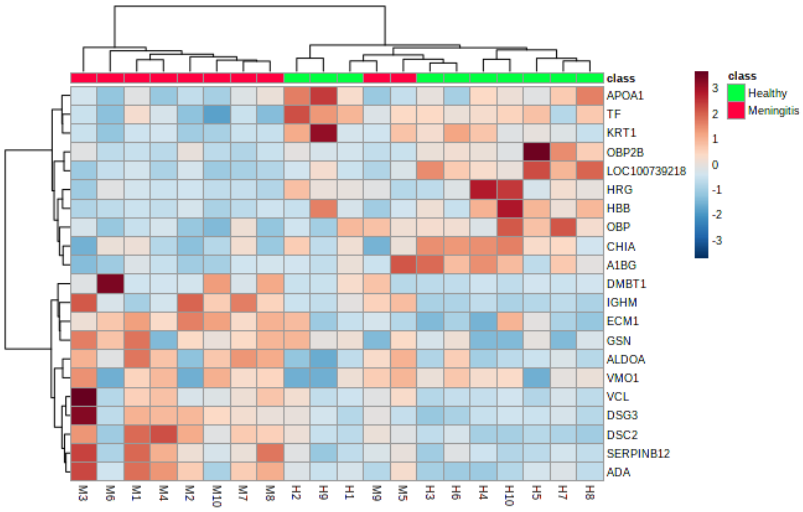
humans (Reams et al., 1994; Greenwood et al., 1997; Bhuiyan et al., 2009; Brodehl et al., 2017).

- ADA, a more established biomarker for inflammatory and immune disorders in pigs, was also upregulated in meningitis and selected to validate the proteomic results due to the availability of an automated spectrophotometric assay validated for pigs (Kaiser et al., 2018; Tecles et al., 2018). This protein did not increase in the serum, confirming the previously documented different behavior in these two fluids (Contreras-Aguilar et al., 2020).
- ALDOA was also found in the saliva of pigs with meningitis, in accordance with the recent report also included in this PhD thesis in which increases in ALDOA were detected in the saliva of pigs in the septic model induced by the administration of LPS (López-Martínez et al., 2022b), and in previous studies in the serum of pigs with meningitis experimentally induced by *S. suis* (Jiang et al., 2020).

- 10 proteins had lower relative abundance in piglets with meningitis. Among the proteins with the lowest FC, the most relevant were:
  - Lipocalin cytosolic FA-bd domain-containing protein (OBP2B), a member of the lipocalin family secreted by mandibular and submandibular glands in pigs (Prims et al., 2019). Its deficiency could lead to high susceptibility to worsening sepsis (Srinivasan et al., 2012).
  - Hemoglobin subunit beta (HBB), the major heme protein of erythrocytes, whose increases in serum have been observed in pigs with sepsis induced by LPS administration (Olumee-Shabon et al., 2020) and were considered an early predictor of sepsis in humans (Yoo et al., 2015). However, in our study, the HBB was decreased in the saliva of pigs with meningitis, reported previously in the saliva of other animal species, like horses with acute abdominal disease (Muñoz-Prieto et al., 2021). Our data could indicate a divergence in the behavior of HBB between saliva and serum in this condition that should be further explored.

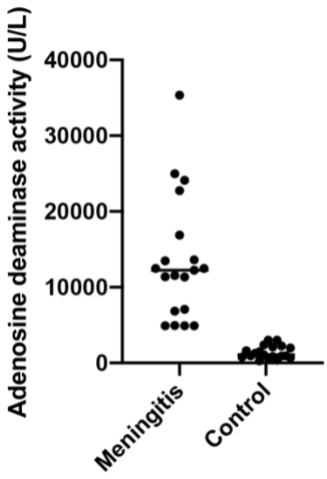
Fig. 18. *Hierarchical cluster analysis based on the proteins with different relative abundance in the saliva of the piglets with meningitis (red board) and the healthy piglets (green board). The red color represents the increased relative abundance, and the blue corresponds to the decreased relative abundance*

in meningitis versus the healthy group. Abbreviations: A1BG—Alpha-1B-glycoprotein, ADA—Adenosine aminohydrolase, ALDOA—Fructose-bisphosphate aldolase, APOA1—Apolipoprotein A-I, CHIA—Chitinase, DMBT1—Isoform 2 of Deleted in malignant brain tumors 1 protein, DSC2—Desmocollin 2, DSG3—Desmoglein 3, ECM1—Extracellular matrix pro- tein 1, GSN—Actin-depolymerizing factor, HBB—Hemoglobin subunit beta, HRG—Cystatin do- main-containing protein, IGHM—Immunoglobulin heavy constant mu, KRT1—Cytokeratin-1, LOC100739218—Double-headed protease inhibitor, submandibular gland-like, OBP—Odorant- binding protein, OBP2B—Lipocln_cytosolic_FA-bd_domdomain-containing protein, SERPINB12— SERPIN domain-containing protein, TF—Serotransferrin, VCL—Metavinculin, VMO1—Vitelline membrane outer layer protein 1.



In the validation of the proteomic results with an automated method, ADA demonstrated excellent performance in the differentiation between healthy and pigs with meningitis due to *S. suis*, showing significantly higher activity levels in the diseased pigs (p<0.001, Fig. 19) and an area under the curve of 0.983 in the ROC analysis.

Fig. 19. *Adenosine deaminase (ADA) activity of saliva in the meningitis group compared with control. The plots show medians (line within box), 25th, and 75th percentiles (boxes), and min and max values (whiskers). Asterisks indicate statistically significant differences.*



Among the proteins that changed in the serum of pigs with meningitis, there were 20 proteins whose relative abundance changed:

- 7 proteins were upregulated, being the most notable:
    - Hp, a moderate APP in swine that has been previously described as increased in inflammatory and infectious processes such as the administration of LPS, but also viral infections (Gómez-Laguna et al., 2010; Olumee-Shabon et al., 2020).
    - Serum amyloid P-component (APCS), a glycoprotein that belongs to the family of pentraxins closely related to CRP, whose main features lie in the modulation of the humoral innate immune system spanning the complement system, inflammation, and coagulation (Poulsen et al., 2017).
    - Three serpin domain-containing proteins, of which two were previously reported upregulated in the serum of pigs with sepsis in one of the studies of this PhD thesis (López-Martínez et al., 2022b).

- 13 proteins were downregulated, being the most protruding changes in:
    - Histidine-rich glycoprotein (HRG), a glycoprotein of particular importance in bacterial infection due to its ability to bind these pathogens, whose reduction could suggest a depleted ability to combat bacterial infections (Kacprzyk et al., 2007; Wake, 2019). In addition, HRG is also involved in the platelet degranulation pathway; therefore, its decrease may lead to a hypercoagulative state, fibrinolysis, and enhanced immune response, ordinary events that occur in sepsis (Kuroda et al., 2018).
    - Apolipoprotein A-I (APOA1), a negative APP and the major protein of high-density lipoprotein (HDL) that has anti-inflammatory and antithrombotic properties (Burger & Dayer, 2002). This protein could be a good predictor of the severity or potential complications in infectious diseases in pigs, as its levels drop fast when infection occurs (Heegaard et al., 2011).

Overall, meningitis caused by *S. suis* resulted in protein changes mainly related to altered platelet and neutrophil pathways. However, although a similar number of proteins changed in both fluids, the proteins were markedly different, manifesting different pathophysiological mechanisms and marking new potential biomarkers for this infection.

3.2.3. Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by Escherichia coli (Article 4)

- Aims and experimental design

This study aimed to identify changes in the salivary proteome of pigs with diarrhoea caused by *Escherichia coli*, the most frequent causal agent of diarrhoea in pigs. Saliva was the chosen biological sample due to its non-invasive nature and its ease to collect in pigs. In addition, saliva can provide complementary information about the pathophysiology of diseases, being a potential source of biomarkers. Saliva samples were collected from two groups of Large White weaning pigs, 10 pigs with clinical signs compatible with this disease (diarrheic syndrome) and positive for the presence of *E. coli* in rectal swabs and *E. coli* F4 and heat-labile toxin; and 10 matched healthy controls. SDS-PAGE (1DE) and two-dimensional gel electrophoresis (2DE) were performed, and significantly different protein bands and spots between groups were identified by mass spectrometry.

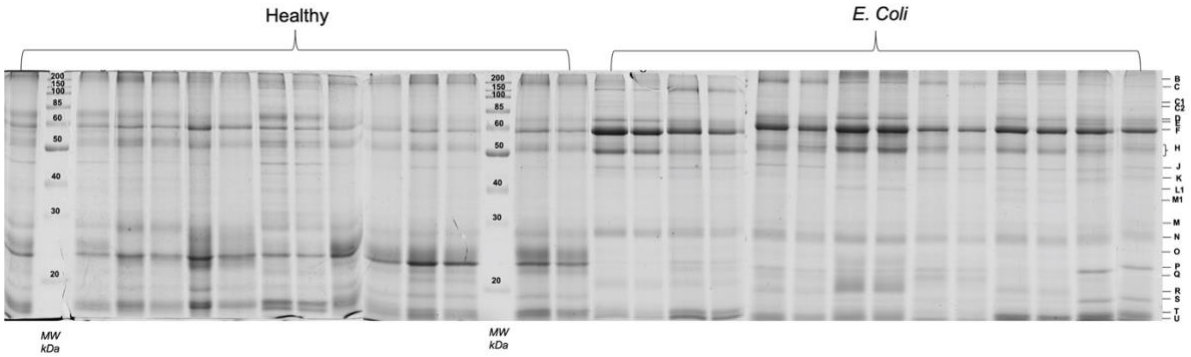
Additionally, the results of one of the proteins that changed in proteomics (ADA) was confirmed with a commercial automated assay in a larger number of pigs (n=28 healthy, n=28 diseased) from the same age and sampled by the same approach as in the proteomic study.

- Results and discussion

The total protein concentration of saliva samples was significantly higher in *E. coli* animals than in healthy ones. Mean *E. coli* animals had almost 3 times higher total protein values than healthy animals (p=0.001).

Salivary SDS-PAGE protein profiles allowed the constant visualization of clearly distinct 21 protein bands, with molecular masses ranging from 10 to 200 kDa, whose levels were subsequently compared between groups (Fig. 20). From those 21 protein bands, 8 were differently expressed between healthy and diseased pigs. Band C1 was a faint band only observed in the *E. coli* group, which could not be identified through mass spectrometry. The other 7 protein bands were present in the pigs from both groups, and showed statistically significant differences, with an increase in the bands B, H, M, N, and R and a decrease in bands P and T in pigs with diarrhoea due to *E. coli* compared to healthy pigs.

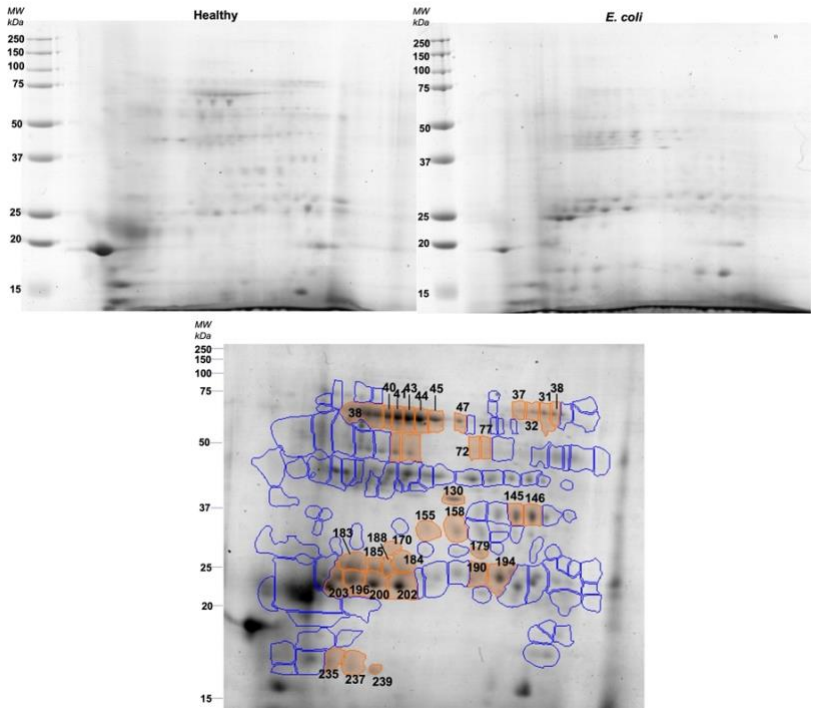
Fig. 20. *Salivary protein profiles (SDS-PAGE) from all the samples (healthy controls and E. coli diseased pigs). Each capital letter on the right side represents the bands compared between groups.*



In SDS-page, increases in lipocalin and IgA bands were observed in diseased pigs, whereas bands containing proteins such as odorant-binding protein or prolactin-inducible protein showed decreased concentrations.

In 2DE, it was possible to consider 127 protein spots in the different pool samples, which were compared between healthy and *E. coli* sample pools, resulting in 35 protein spots with a statistically significant difference. Among these, 15 protein spots increased in *E. coli* animals, whereas 20 decreased (Fig 21).

Fig. 21. *Representative gels of healthy (upper left) and E. coli (upper right) pools. The lower image represents the reference gel with protein spots differently expressed between groups (orange) and spots that did not show differences between groups (blue).*

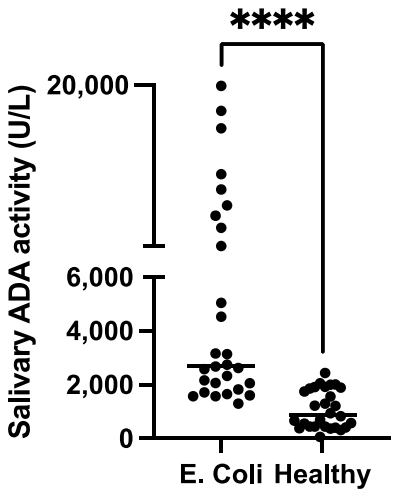


Overall, in the two proteomic techniques, saliva from pigs with *E. coli* presented higher expression levels of:

- Lipocalin (LCN), a family of proteins expressed in numerous tissues and involved in multiple processes, including inflammation and immune activation. Lipocalin-2 is an APP that can capture bacterial siderophores produced by pathogenic bacteria such as *E. coli* (Abella et al., 2015; Moschen et al., 2017; Wang et al., 2021).
- IgA, an immunoglobulin found in large amounts in the mucosal secretions of the gastrointestinal tract and saliva, which is produced by the immune system to prevent the invasion of pathogenic microbes. This results could agree with other reports that described an increase in IgA in mucosal secretions after an *E. coli* infection (Pietrzak et al., 2020).
- Albumin peptides, which could indicate a proteolysis of the whole albumin in the saliva of diseased pigs, a fact that has been previously described in the blood in other diseases such as renal failure.
- ADA activity, whose increases have been reported previously in inflammation and sepsis in the saliva of pigs, including a study contained in this PhD thesis (Contreras-Aguilar et al., 2020; López-Martínez et al., 2022a). This analyte was the chosen biomarker to validate proteomic results.

The measurement of ADA with a previously validated commercial automated assay showed higher concentrations of salivary ADA activity in the pigs with diarrhoea to *E. coli* compared to healthy pigs ($p<0.001$, Fig. 22).

Fig. 22. *Comparison of the salivary ADA in pigs with diarrhoea caused by E. coli and healthy pigs. The plot shows the individual values of each group. ****p<0.001.*



On the other hand, considering the two proteomic techniques, saliva from pigs with *E. coli* presented lower expression levels of:

- Odorant binding protein (OBP), involved in olfaction and the defense against oxidative injury and inflammation. Its decreased levels could be explained by the activation of an additional mechanism that would allow inflammatory mediators to stimulate neutrophil recruitment and oxidative burst in the lung and possibly in other tissues (Mitchell et al., 2011).
- Prolactin-inducible protein (PIP), related to the immune response and inhibition of the growth of bacterial species. Its decrease could be related to a drop in prolactin, described in pigs with inflammation and humans with sepsis (Hassan et al., 2009; Elmasry et al., 2016; Kaiser et al., 2018).
- Alpha-amylase, involved in the sympathetic nervous system activation. Usually, the alpha-amylase activity in pig saliva is increased in stress and disease; this divergence could be explained because the 2DE spots represent the relative amount of the forms of the protein, which may not be the most contributing ones concerning the enzymatic activity (Contreras-Aguilar et al., 2017, 2019).
- Carbonic anhydrase VI, representing a group of enzymes that catalyze the reversible hydration/dehydration of CO_2 and water. Its decrease could be related to damage in the intestinal mucosa (Oikonomou et al., 2012).
- Whole albumin, usually decreased in critically ill patients, in which hypoalbuminemia is a significant cause of higher mortality rates (Artigas et al., 2016).

In this report, pigs with diarrhoea caused by *E. coli* had changes in proteins in their saliva related to various pathophysiological mechanisms such as inflammation and immune function in a similar way to other proteomic studies included in this PhD thesis in pigs after LPS-induced sepsis and other infectious diseases such as meningitis due to *S. suis*. These proteins could be potential biomarkers in diagnosing and monitoring diarrhoea caused by *E. coli* infection.

3.3. Objective 3

Objective 3 was covered by three studies corresponding to articles nº 5 to 8 and Experiment 1 in the Annex, in which it was performed the validation and measurement of various biomarkers of inflammation, oxidative stress, welfare, and muscle damage, with potential application in septic inflammation and study of their possible changes in sepsis and other conditions.

3.3.1. Salivary D-dimer in pigs: Validation of an automated assay and changes after acute stress (Article 5)

- Aims and experimental design

This research aimed, firstly, to evaluate if the concentrations of D-dimer could be measured in pig saliva and, secondly, to assess whether D-dimer concentration changes in porcine saliva after an acute stress stimulus. For this purpose, complete analytical validation of a commercial immunoturbidimetric D-dimer assay in saliva samples was performed, and an experimental acute stress model was induced in 11 Large White pigs in the mid-fattening period through restraint by nose-snare immobilization for 1 min. Saliva samples of the pigs were collected at different times (TB: before the restraint, baseline time; T0: during the restraint; and T15 and T30: 15 and 30 min after the restraint) and changes in D-dimer concentrations were evaluated. The more usual stress biomarkers cortisol and sAA were also evaluated for comparative purposes.

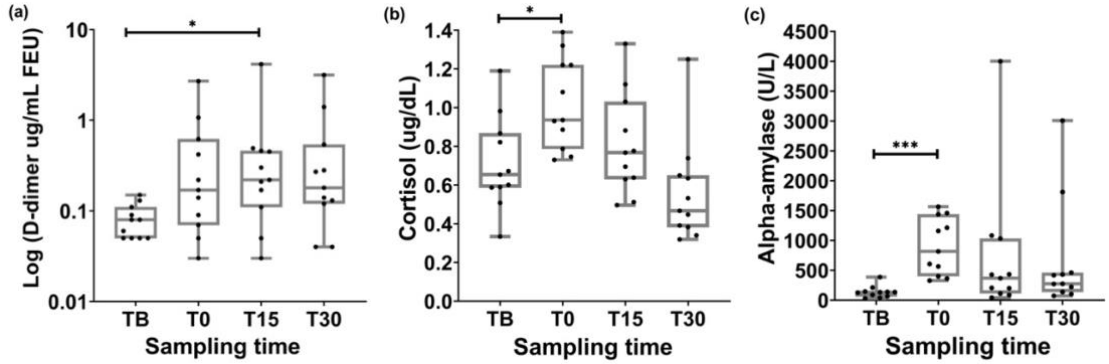
- Results and discussion

Regarding the analytical validation of the method, intra and inter-assay CVs were, in all cases, <8%. The LOD of the method was set at 0.129 mg/mL FEU, and the LLOQ at 0.167 mg/mL. Linear regression equations of observed (measured) salivary D-dimer concentration (X-axis) vs expected salivary D-dimer concentration (Y-axis) showed r^2 >0.98. A range from 95.01% to 106.88% was observed in the recovery test.

In the experimental acute stress model responses, changes in D-dimer, cortisol, and sAA values after the nose-snare immobilization are presented in Fig. 23 (a, b, c). After the experimental acute stress model, there was a significant increase in D-dimer at T15 compared to TB (p=0.0496). Cortisol showed a significant increase at T0 compared to TB (p=0.0496). Salivary alpha-amylase (sAA) also showed a significant increase at T0 compared to TB (p=0.0002). The Spearman correlation test evidenced a positive

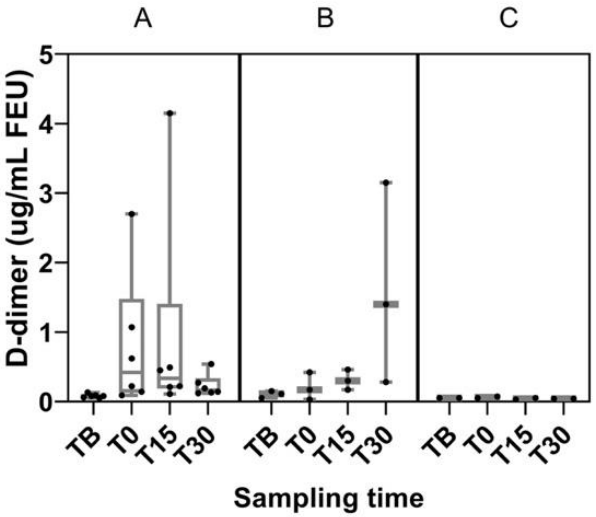
moderate correlation between D-dimer and sAA (p=0.0002). No correlation between D-dimer and cortisol (p=0.0716) was observed.

Fig. 23. *D-dimer(a), cortisol(b) and salivary alpha amylase(c) values in saliva obtained from the model of experimental acute stress. TB, basal time; T0, during the restraint; T15 and T30, 15 and 30 min after the restraint. Graphs show medians (line within box), 25th and 75th. percentiles (boxes), min and max values (whiskers) and individual values (points). Asterisks indicate statistically significant differences (*p<0.05). In Fig. 23a, the Y-axis (D-dimer μg/mL FEU) is shown on a logarithmic scale.*



The salivary D-dimer concentration increase occurred at different times depending on the animal (Fig. 24). A fast increase was observed in six pigs, with a maximum peak of D-dimer concentration found at T0 or T15. In comparison, the major increase in D dimer concentration was found at T30 in three pigs, and the other two pigs showed no change in D dimer concentration.

Fig. 24. *Salivary D-dimer concentrations found at different times among individual pigs. A rapid increase was observed in 6/11 pigs with a peak at T0 or T15 (A), while 3/11 pigs had the D-dimer peak at T30 (B), and 2/11 pigs had no increase in D-dimer (C). TB, basal time; T0, during the stressor application; T15 and T30, 15 and 30 min after the stressor application. Graphs show medians (line within box), 25th and 75th. percentiles (boxes), min and max values (whiskers) and individual values (points).*



To the authors' knowledge, this is the first report in which D-dimer has been analyzed in saliva samples of any veterinary species, and the analytical validation results were similar to those of a previous study in which D-dimer was measured in human saliva with an AlphaLISA assay (Zhang et al., 2013).

D-dimer concentrations increased in the saliva of pigs after the experimental acute stress model. The positive correlation of D-dimer concentrations with sAA activity and the lack of correlation with cortisol suggests that this rise could be associated with the autonomic nervous system response to stress, which would be consistent with previous studies performed in humans and mice that found that this system can activate blood clotting after a stressful event (Stämpfli et al., 2014; von Känel et al., 2019).

The peak concentration of D-dimer was observed at different times (T0, T15 or T30) depending on the pig. Similarly, von Känel et al. (2019) stated that the higher D-dimer levels in human blood following a stress stimulus occurred at different time points in each person. They hypothesized that chronic stress could impair fibrinolytic activity, leading to a slower breakdown of blood clots and subsequently causing a delayed release of D-dimer (Von Känel, 2015). Further studies would be of interest to evaluate whether previous exposure of pigs to chronic stress can influence the response of D-dimer after an acute stressful event.

3.3.2. Changes in biomarkers of redox status in saliva of pigs after an experimental sepsis induction (Article 6)

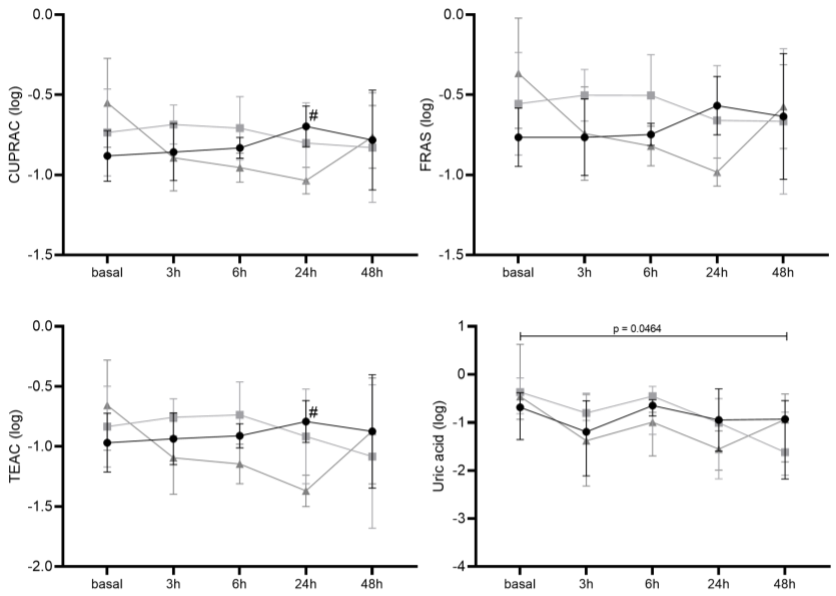
- Aims and experimental design

This study aimed to evaluate the changes that can occur in biomarkers of the redox status in the saliva of pigs with experimentally induced sepsis. To this end, several redox status biomarkers were measured in the saliva of pigs with experimentally induced sepsis by endotoxin lipopolysaccharide (LPS) administration, non-septic inflammation induced by turpentine injection, and in healthy individuals with NaCl. Saliva was collected in the pig before and after 3, 6, 24, and 48 h. The biomarkers measured were the antioxidants cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of saliva (FRAS), trolox equivalent antioxidant capacity (TEAC), and acid uric; and the oxidants advanced oxidation protein products (AOPP), ferrous oxidation-xylenol orange (FOX), peroxide activity (POX-Act), and reactive oxygen-derived compounds (d-ROMs).

- Results and discussion

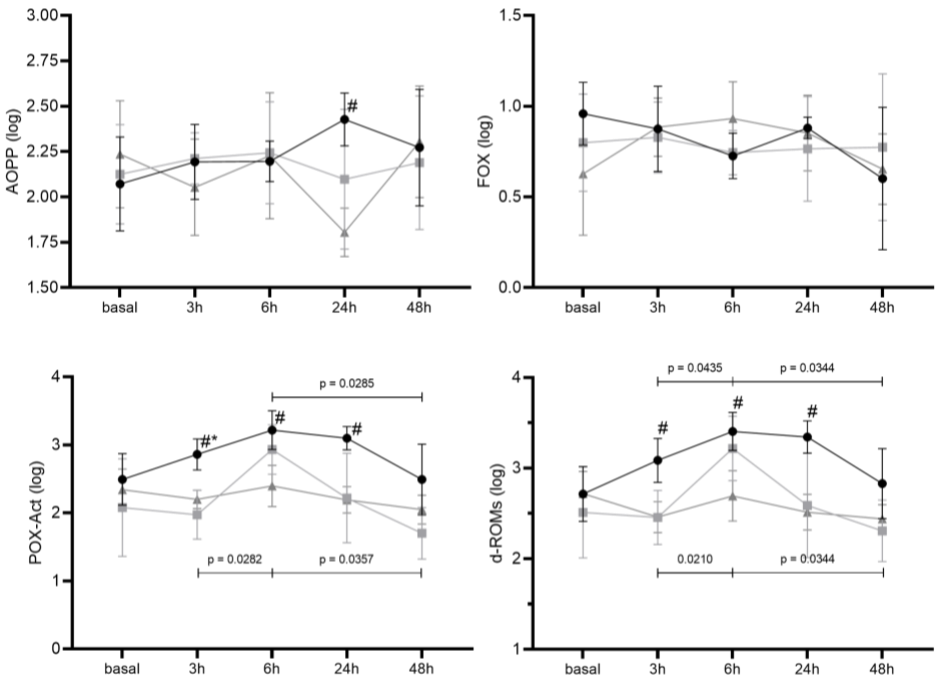
Most antioxidant biomarkers measured in this study were higher in LPS-treated pigs compared to the control group. The group of pigs treated with LPS had significantly increased concentrations of CUPRAC 24 h after treatment when compared to basal time (p=0.039, Fig. 25a). TEAC displayed a tendency to increase (p=0.056, Fig. 25b) at 24 h in comparison to basal time. When compared to the healthy control group, the LPS group showed significantly higher concentrations of CUPRAC (p=0.004; Fig. 25a), FRAS (p=0.028; Fig. 25b), and TEAC (p=0.001; Fig. 25c) 24 h after treatments. In the group of pigs treated with TURP, no changes in antioxidant biomarkers were observed throughout the study, except for uric acid (Fig. 25d), which decreased at 48 h compared to the basal time (p=0.044). No significant difference was evidenced when comparing the control and TURP group at any time point (p>0.05, Fig. 25). The origin of the increases in oxidant biomarkers in sepsis could be the activation of the phagocytic NADPH oxidase complex and the subsequent production of reactive nitrogen and oxygen species (RNS and ROS, respectively). The massive and sustained production can cause damage to endothelial cells and even organ failure, which is associated with increased morbidity and mortality in sepsis patients (Draganov et al., 2010).

Fig. 25. *(a) CUPRAC, (b) FRAS, (c) TEAC and (d) uric acid concentrations in control (45▲), LPS (●), and TURP-treated pigs (■) before (basal) and 3, 6, 24 and 48 h after treatments. The results are presented as a median with an interquartile range. #, significantly different from the control group (p<0.05; one-way ANOVA with Sidak's multiple comparisons test). Bars and the obtained p-value (one-way ANOVA with Tukey's multiple comparison test) indicate differences between times.*



Regarding the oxidant biomarkers, most were also higher in the pigs induced with sepsis than in healthy animals. POX-Act (Fig. 26c) and d-ROMs (Fig. 26d) were significantly higher in LPS-treated pigs than in the control group at 3 h (p<0.039), 6 h (p<0.033) and 24 h (p<0.001), reaching the highest values at 6 h. POX-Act was higher in the LPS group than in the TURP-treated group at 3 h (p<0.036). The LPS group showed significantly higher concentrations of AOPP 24 h after the injections when compared to controls (p=0.004, Fig. 26a). No significant differences were evidenced in FOX (p>0.05, Fig. 26b). The increased antioxidant response in the LPS-induced pigs could be an attempt to compensate the potential damage produced by the overproduced oxidants observed in this group. In fact, α-tocopherol and ascorbic acid, antioxidants that are measured by the assays CUPRAC, TEAC and FRAS from this study, participate in the first line of defense against intravascular oxidants in sepsis (Doise et al., 2008).

Fig. 26. *Salivary (a) AOPP, (b) FOX, (c) POX-Act and (d) d-ROMs concentrations in control (46▲), LPS (•), and TURP-treated pigs (■) before (basal) and 3, 6, 24 and 48 hours after treatments. The results are presented as a median with an interquartile range. #, significantly different from the control group (p<0.05; one-way ANOVA with Sidak's multiple comparisons test). Bars and the obtained p-value (one-way ANOVA with Tukey's multiple comparison test) indicate differences between times.*



In general, this study reports an increase in oxidant and antioxidant markers during induced sepsis by LPS administration in pigs, revealing changes in redox status in pigs with this condition. In the animals with local aseptic inflammation, the changes in redox

biomarkers were of a lower magnitude. Therefore, biomarkers of redox status can potentially evaluate septic and non-septic inflammation conditions in swine.

3.3.3. Novel saliva biomarkers for stress and infection in pigs: changes in oxytocin and procalcitonin in pigs with tail-biting lesions (Article 7)

- Aims and experimental design

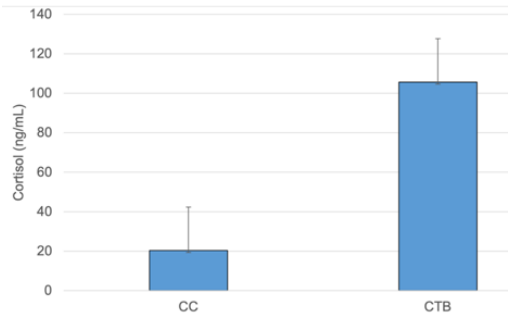
The current study sought to evaluate oxytocin and procalcitonin, two analytes of animal welfare and health, in a spontaneous tail-biting outbreak. Tail biting is a problematic behavior that causes stress and an increased risk for infections in the pig. Oxytocin and PCT were compared with more traditional biomarkers related to stress, inflammation, and immune activation, namely cortisol, Hp and ADA with its isoenzymes.

Growing pigs from a commercial farm were selected, classifying them as control pigs from control pens (CC, n=30), control pigs within tail-biting pens (CTB, n=10) and pigs with fresh tail lesions from pens with tail-biting outbreak (LTB, n=27). Some of the animals with tail lesions had mild lesions (MTB, n=13), and other had severe lesions (STB, n=5). A saliva sample was collected from each pig to be analyzed for the stated biomarkers related to stress, infection, inflammation, and immune activation.

- Results and discussion

The only biomarker that was significantly higher in CTB than in CC pigs was cortisol (p<0.001, Fig. 27). It is necessary to consider that in the CTB group minor signs of lesions appeared, and the pigs with no lesions were usually the only ones in the pen, which suggest that they could be the biters or could change their normal behavior to stay neutral, such as by reducing feed intake, which could also be stressful (Palander et al., 2013).

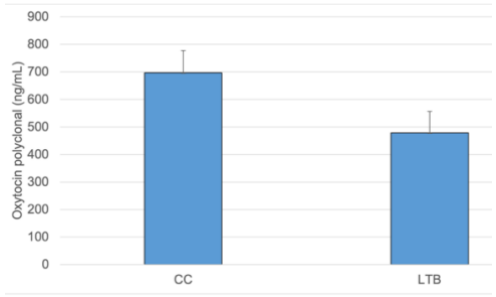
Fig. 27. *Estimated marginal means and standard error for cortisol (ng/mL) in control pigs from control pens (CC) and control pigs from tail-biting pens (CTB). The phenotypes differ significantly (p<0.001).*



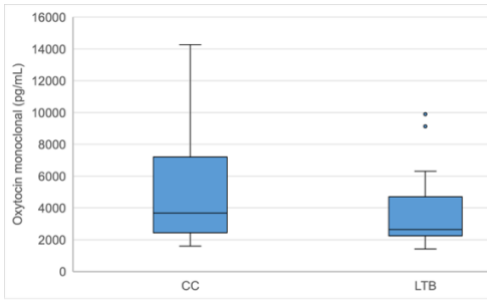
Oxytocin concentrations tended to be higher in CC pigs than in LTB pigs (p=0.06, Fig. 28a,b), suggesting that these pigs may suffer more stress.

Fig. 28. *(a) Estimated marginal means and standard error for polyclonal oxytocin (ng/mL) and (b) boxplot based on original values for monoclonal oxytocin (pg/mL) in control pigs from control pens (CC) and lesioned pigs from tail-biting pens (LTB). The phenotypes tended to differ for both oxytocin measures (p=0.06).*

a.



b.



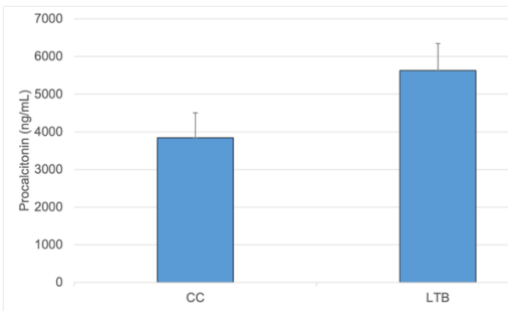
PCT showed a tendency to be higher (p=0.07) in LTB than in CC (Fig. 29a), which suggests a potential spread of bacteria from the tail lesions to the bloodstream (Sihvo et al., 2012). In humans, PCT can identify severely infected ulcers (Jeandrot et al., 2008), but further studies are needed to establish PCT ranges for confirming infection in pigs.

Hp was higher in LTB than in CC pigs (p<0.001, Fig. 29b), as was expected, since Hp is a validated marker of inflammation, trauma, and infection (Cerón et al., 2022). In addition, Hp has been suggested as a biomarker for stress (Salamano et al., 2008).

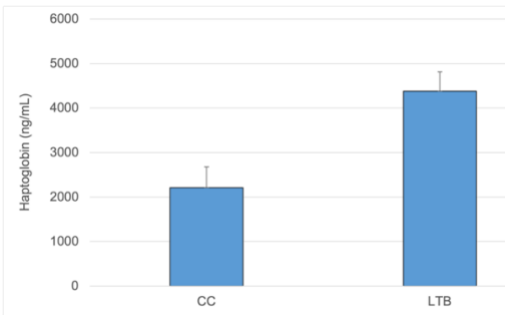
ADA and its isoenzymes did not change significantly between groups (p>0.01). However, ADA2 correlated with Hp, oxytocin and cortisol, especially in pigs from TB pens, which could suggest a relationship between ADA and stress, as previously reported in lame and prolapsed pigs (Contreras-Aguilar et al., 2019).

Fig. 29. *(a) Estimated marginal means and standard error for PCT (ng/mL) and (b) Hp (ng/mL) in control pigs from control pens (CC) and lesioned pigs from tail-biting pens (LTB). The phenotypes tended to differ for PCT (p=0.07) and differed significantly in Hp levels (p<0.001).*

a.



b.



Finally, correlations between all biomarkers of the different groups (CC, CTG, and LTB) were mainly moderately positive, highlighting the correlation between oxytocin and cortisol like in some previous studies (Brown et al., 2016), and a strong correlation between oxytocin and procalcitonin only in CC pigs that needs further research.

The tendency of higher oxytocin levels in CC pigs than in LTB pigs and PCT in LTB and CC pigs could suggest a relationship between sepsis and stress. Furthermore, in previous studies, increases in oxytocin have been observed in sick animals in compensatory situations to inhibit inflammation (Işeri et al., 2005) and, therefore, this hormone could play an important role in immune modulation of events associated with sepsis and limitation of organ damage (Sendemir et al., 2013). Further studies are needed to clarify the role of these biomarkers in tail-biting in pigs.

3.3.4. Measurement of Calprotectin (S100A8/A9) in the Saliva of Pigs: Validation Data of a Commercially Available Automated Assay and Changes in Sepsis, Inflammation, and Stress (Article 8)

- Aims and experimental design

This study aimed to validate an automated assay for calprotectin (CALP, S100A8/A9) measurement in porcine saliva. CALP is involved in a wide range of proinflammatory functions and, thus, is used as a biomarker of inflammation and sepsis in faeces, serum, and saliva in humans. In pigs, CALP concentrations have been measured in faeces from animals with colitis (Bogere et al., 2019; Barbosa et al., 2021), but to the authors' knowledge, there are no reports about its measurement in porcine saliva.

A complete analytical validation of an automated commercial kit of calprotectin was performed, and the concentrations of this biomarker were evaluated in three situations:

- Different times of sampling: saliva samples from 20 finishing male Large White pigs from a previous study were used (Ortín-Bustillo et al., 2022), being the samplings performed on the same day at 8 a.m., 12 a.m., 4 p.m., and 8 p.m.
- In septic and non-septic inflammatory conditions: samples from the model of LPS and TURP described in the general methods section were used. Saliva and blood samples were measured at TB, T6, T24, and T48.
- In stressful conditions: a model of transport from the farm to a commercial slaughterhouse. A total of 13 Large White pigs at the end-fattening period were

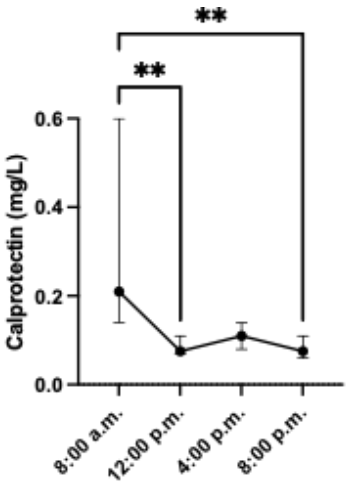
included, and saliva samples were collected upon arrival at the slaughterhouse on the day of transport (T0) and after 4 h, following the placement in the lairage area (T4).

- Results and discussion

The analytical validation of the method in saliva resulted in intra and inter-assay CVs of <5%, recovery rates ranging from 110% to 116.7%, and linear regression equations with a coefficient of correlation close to 1 in serially diluted samples. The LLOQ was set at 0.01 mg/L for salivary CALP, and the LOD of the assay could not be calculated since all measurements with ultrapure water gave a value of zero.

CALP concentrations in saliva showed a tendency to decrease during the day; these changes were significant at 12 and 8 p.m. when compared with values at 8 a.m. (Fig. 30).

Fig. 30. *Results of salivary calprotectin concentrations in 20 male pigs obtained at different hours of the day. **p<0.01. Dots represent the median values and whiskers the 95% CI.*



In the experimental septic and non-septic inflammation induction:

- The salivary CALP concentrations were significantly higher in LPS-induced pigs at T24 compared to TB (p=0.005) and T48, in which CALP concentrations were already close to basal levels. In the TURP group, no significant changes were obtained, although a tendency for higher concentrations in T6 was observed. There were no changes in the control group (Fig. 31).

- In serum, LPS-induced pigs showed a significant increase at 6 h compared with baseline values (p=0.01). In the TURP group, no significant changes were obtained in the pairwise comparison, although a tendency of increase at 6 h and 24 h compared with the control group was observed (Fig. 32).

Figs. 31 and 32. *Changes in the salivary (31) and serum (32) calprotectin concentrations of pigs after LPS, TURP or saline (control) injection. The plots show medians (line within box), 25th and 75th percentiles (boxes), and min and max values (whiskers). Asterisks indicate a statistically significant difference: **=p<0.01.*

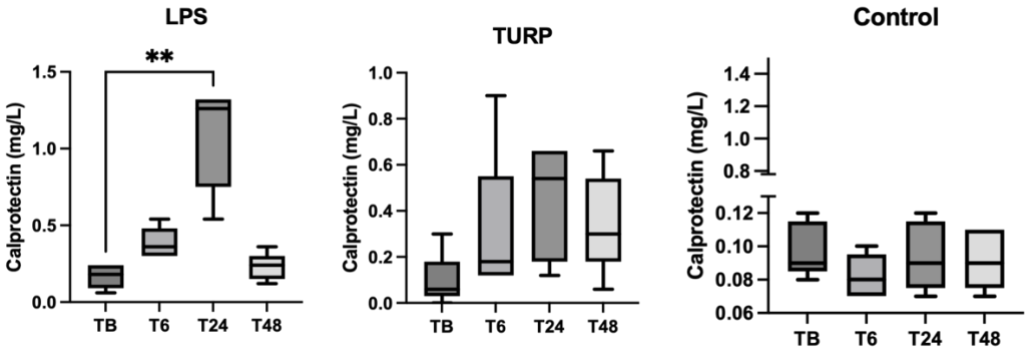


Fig. 31.

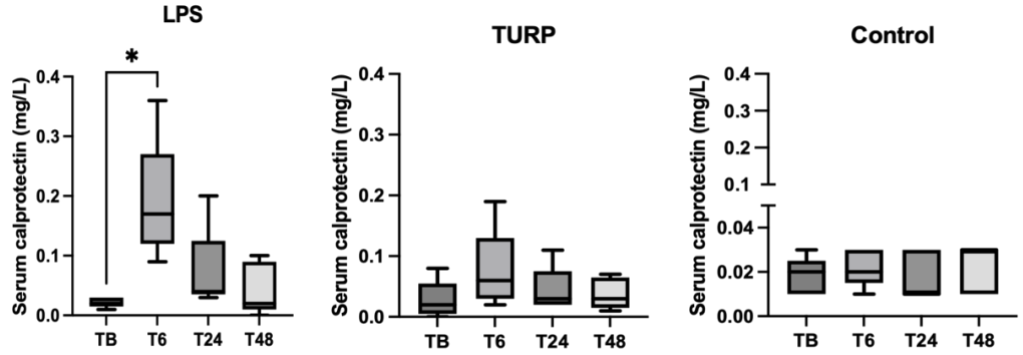
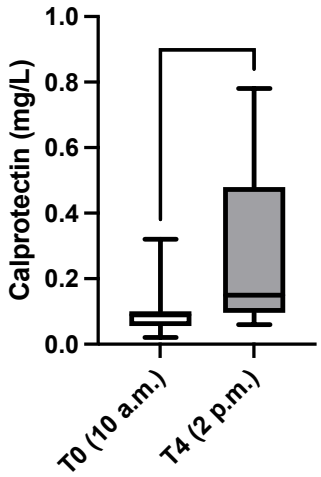


Fig. 32.

In the model of stress, CALP was significantly higher in pigs 4h after arrival at the slaughterhouse compared with T0 values (p=0.002, Fig. 33).

Fig. 33. *Concentrations of calprotectin in pigs at the arrival to the slaughterhouse (T0) and 4 h after transportation (T4). The plots show medians (line within box), 25th and 75th percentiles (boxes) and min and max values (whiskers). **=p-value<0.01.*



Therefore, CALP can be measured in pigs' saliva, and its concentration showed variations depending on the time of the day in which the sample was collected. In addition, CALP increased in the saliva of pigs with LPS-induced sepsis more than in pigs with induced non-septic inflammation. Moreover, this protein increased after a stressful situation in a model of transport in pigs, although these increases were of lower magnitude than the ones observed in sepsis. Therefore, CALP could be a potential biomarker of health and welfare in swine.

3.3.4. Changes in Salivary Biomarkers of Stress, Inflammation, Redox Status, and Muscle Damage due to Streptococcus suis Infection in Pigs (Experiment 1 in Annex)

- Aims and experimental design

The hypothesis of this study was that saliva could reflect changes in different biomarkers in an *S. suis* infection and that they could help diagnose the disease.

To this end, a panel integrated by analytes to evaluate stress, septic and aseptic inflammation, redox status, and muscle damage was performed. This panel consisted of cortisol, alpha-amylase (sAA) and oxytocin (OXT) as biomarkers of stress; haptoglobin (Hp) and total protein as indicators of inflammation; the ferric-reducing ability of saliva (FRAS) and advanced oxidation protein products (AOPP) as biomarkers of redox status; creatine kinase (CK), CK-myocardial band (CK-MB), troponin I, lactate, lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) to assess possible muscle damage; and calprotectin (CALP, S100A8-A9) and calgranulin C (S100A12) to evaluate inflammation and sepsis. In addition, three proteins related to the immune system, inflammation, and sepsis (ADA, PCT and ALDOA) that were confirmed to increase in *S. suis* infection in studies included in this PhD thesis (López-Martínez et al., 2022a; López-Martínez et al., 2022b; López-Martínez et al., 2022c) were also evaluated.

A total of 56 Large White male growing pigs from a farm were selected as the meningitis group (n=28) and healthy group (n=28).

The criteria to select the pigs of the meningitis group were: 1) having clinical signs compatible with *S. suis* infection, 2) not having been treated before, and 3) being positive for *S. suis* at the analytical diagnostic tests. The pigs with meningitis tested positive for *S. suis* serotype 9 by a polymerase chain reaction (PCR) based on the glutamate

dehydrogenase gene. The severity of the disease in the infected pigs was classified on a five-point scale (1-No clinical signs, 2-Mild disease, 3-Moderate-low disease, 4-Moderate-high, 5-Severe disease) based on the presence of hyperthermia, arthritis, and two grades of neurological signs (Table 2).

***Table 2**. Severity scale in pigs infected by S. suis. – No presence; + Moderate; ++ Severe.*

Grade of severity	Description	Hyperthermia	Arthritis	Neurological manifestations
1	No clinical signs	-	-	-
2	Mild disease	-	-	+
3	Moderate	+	-	+
4	High	+	+	+
5	Severe disease	+	+	++

- Results and discussion

Results showed significant increases in biomarkers related to:

- Stress: sAA, OXT and cortisol, with a 9, 1.8 and 1.7-fold increase in meningitis. No previous report was found about changes in cortisol in any species due to *S. suis* infection, but this biomarker has been shown to increase in other infectious diseases (Torpy & Ho, 2007). Regarding sAA, its increase could be associated with the pain and discomfort induced by the disease, as described in other pathologies in pigs (Contreras-Aguilar et al., 2019). OXT is a nonapeptide hormone used to assess positive and stressful situations in animals (López-Arjona et al., 2020a) but it has also been shown to have anti-inflammatory effects in the early stages of sepsis (Mehdi et al., 2022), and a possible protective role against sepsis by limiting organ damage associated with this pathology (İşeri et al., 2005; Sendemir et al., 2013).

- Inflammation: Hp, total protein, CALP, and calgranulin C; with a 1.5, 1.9, 3.4 and 3.6-fold increase, respectively. Hp is a moderate APP in swine and has been previously described in the serum of pigs with *S. suis* in another study from this PhD thesis (López-Martínez et al., 2022b) and other inflammatory and infectious processes such as the administration of LPS and viral infections (Gómez-Laguna et al., 2010; C. Yin et al., 2017). The increments of total protein found in the saliva of pigs with meningitis found in this study could be due to the higher protein production in inflammation states, as it was observed in the serum of pigs with bacterial infections due to the increases in several APPs (Heegaard et al., 1998). S100A8-A9 (Calprotectin) and S100A12 (Calgranulin C) are calcium-binding proteins of the S100 family, located in the cytosol of neutrophils and monocytes and released after the

activation of these cells. These two proteins are involved in inflammation and sepsis, having an antimicrobial function (Barbosa et al., 2021), and both showed a high correlation in this study (Spearman r=0.90, p<0.001), like what has been described in human serum (Dubois et al., 2019).

- Redox status: AOPP, with a 1.6-fold increase, a change that was also described in experimentally induced sepsis in pigs in this PhD thesis, possibly due to the liberation of oxidant products during this process (López-Martínez et al., 2022d).

- Muscle damage: CK, CK-MB, troponin I, lactate, LDH, AST and ALT, with a 1.3, 1.9, 1.8, 3.27, 5.4, 2.5 and 2.4-fold increase, respectively. Increases in CK and AST in serum have been observed during transportation, mainly associated with the physical activity accompanying the handling that causes muscle damage in the animals (Golightly et al., 2021). Moreover, AST and ALT are less specific for skeletal muscle than CK because of their presence in the liver (Brancaccio et al., 2010) and, therefore, their increases could also indicate potential liver damage associated with meningitis. Troponins are high-specific cardiac alteration markers in humans (Brancaccio et al., 2010) and animal species such as sheep (Reza Aslani et al., 2013), being previously related to myocarditis associated with infections by *Streptococcus* (Allen et al., 2022). Regarding LDH, its increases can be produced by cardiac damage or by the injury of other muscle types. This study's findings would align with a previous report of this PhD thesis in which increases in salivary VCL, present in the cardiac muscle (Lee et al., 2019), was described in *S. suis* infection in pigs (Wolf et al., 2020). The presence of myocardial damage in the animals with meningitis in our study was also supported by the increments in CK-MB, a diagnostic marker for myocardial damage that was found to increase associated with viral and bacterial infections (Lai et al., 2021; Cersosimo et al., 2022).

In addition, a significant increase in ADA, PCT, and ALDOA in infected animals (with a 9.1, 3.3 and 1.6-fold increase, respectively) was also observed, as previously described in this species and illness.

The ROC curve analyses showed AUCs higher than 0.8 for sAA, total proteins, CALP, calgranulin C, LDH, PCT, AST, and ADA. The grade of severity of the disease indicated a significant positive correlation with total protein concentrations, AST, ALDOA, and AOPP.

This report revealed that *S. suis* infection caused variations in analytes related to stress, inflammation, redox status, and muscle damage in the saliva of pigs, and these can be considered potential biomarkers for this disease.

3.4. Objective 4

Objective 4 was covered by one published study corresponding to article nº 9, and the Experiments 2 and 3 in the Annex. In these articles, the objective was developing and validating new assays to diagnose sepsis: procalcitonin and presepsin.

3.4.1. Measurement of procalcitonin in saliva of pigs: a pilot study (Article 9)

- Aims and experimental design

This study aimed to explore if PCT, a widely used biomarker of sepsis in human medicine, could be measured in the saliva of pigs and whether its concentration changes in sepsis, which would have a huge potential in the veterinary species.

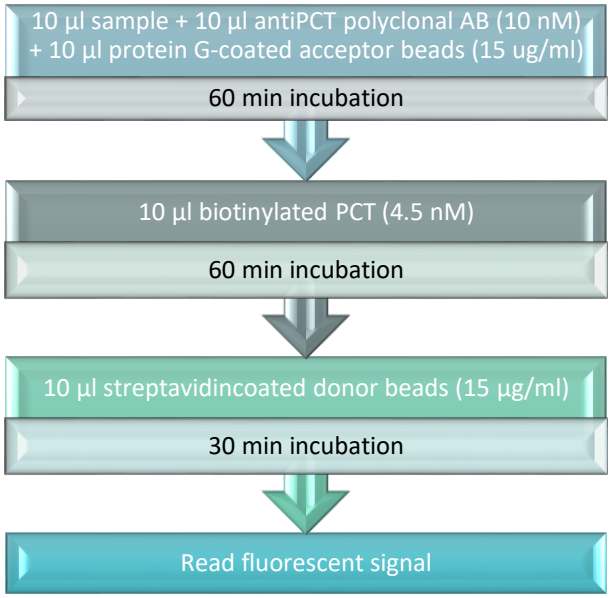
For this purpose, a specific indirect competitive AlphaLISA method with an in-house produced polyclonal antibody was developed, optimized, and analytically validated.

Secondly, changes in PCT concentration were evaluated in two conditions: a) in the experiment of LPS-induced sepsis and turpentine-induced aseptic inflammation, and b) in healthy piglets (n=11) compared to piglets with meningitis (n=20), a disease that usually leads to sepsis and whose treatment often requires large amounts of antibiotics in farms. The chosen pigs with meningitis had as most frequent symptoms ataxia, anorexia, lateral recumbency, paddling, and a median rectal temperature of 40.5 ºC.

- Results and discussion

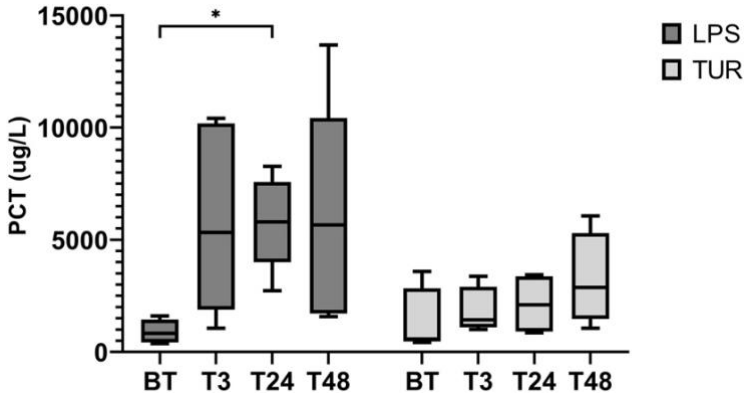
The AlphaLISA protocol of the developed method for PCT measurement in the saliva of pigs is shown in Fig. 34. The assay showed an acceptable precision with CVs within the recommended limits (<20%) and adequate accuracy with linearity after serial sample dilutions with an R^2=0.99 and spike recovery tests between the recommended limits (80-120%). The method's detection limit was set at 68 µg/L, and the lower limit of quantification was 414 µg/L.

Fig. 34. *AlphaLISA protocol for PCT measurement in the saliva of pigs.*



In the LPS/turpentine experiment, higher concentrations of PCT after 24h of the LPS injection (6.35-fold increase) compared to those treated with turpentine oil (1.64-fold increase) were found (p=0.045, Fig. 35). These lower increases in non-septic inflammatory conditions have been described in humans in conditions like severe burns, trauma, or major surgery (Becze, 2016).

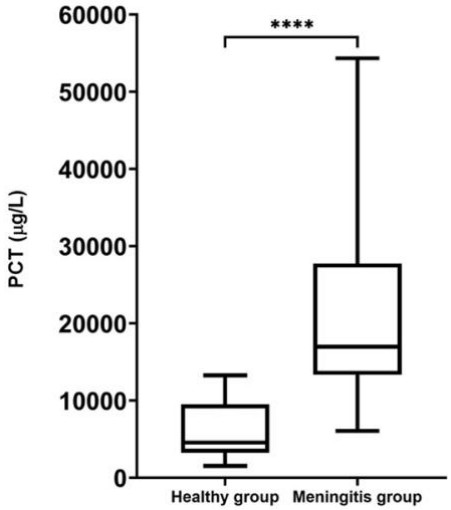
Fig. 35. *PCT concentrations (µg/L) at the evaluated times in the LPS and turpentine oil. BT=basal time; T3, T24 and T48=3, 24 and 48 h after the injections of LPS and oil-turpentine in both groups. Graphs show medians (line within box), 25th and 75th percentiles (boxes), min and max values (whiskers) and individual values (points). Asterisks indicate statistically significant differences ($p<0.05$).*



In addition, PCT was higher in pigs with meningitis (3.53-fold) than in healthy ones (p<0.001, Fig. 36). This magnitude of increase is consistent with previous studies performed in human saliva, in which a 3.45-fold increase was observed in bacterial

exacerbations of Chronic Obstructive Pulmonary Disease (Patel et al., 2015); however, these magnitudes are lower than in the LPS group of our study. PCT concentrations could be influenced by the severity and duration over time of the disease, the health status of the farm, or age differences; for example, PCT average concentrations are higher in human neonates (Eschborn & Weitkamp, 2019) and, in our study, the piglets from the healthy/meningitis trial were younger than the pigs from the LPS model.

Fig. 36. *PCT concentrations (µg/L) in the pigs with meningitis compared with the healthy group. Graphs show medians (line within box), 24th and 75th percentiles (boxes), min and max values (whiskers) and individual values (points). Asterisks indicate statistically significant differences (****=p<0.0001).*



In general, the concentrations of PCT in healthy and septic pigs found in this study were higher than those described in human blood, something previously described also in horses (Rieger et al., 2014), where normally, basal concentrations of PCT are undetectable (Nakamura et al., 2013). As this is the first time PCT has been measured in the saliva of pigs, there are no established reference ranges in this species and type of sample. Although further studies are needed to understand the reason for these different concentrations, one factor could be the influence in circulating PCT of the species-specific quantities of gram-negative bacteria present in the normal intestinal flora. This factor could explain that in previous reports in horses and our study in pigs, basal concentrations of PCT were detectable (Dicks et al., 2014; Costa et al., 2015; Bonelli et al., 2017). Also, the different magnitude of concentration observed could be due to the type of biological sample or immunoassay used. For example, in human saliva, two-fold higher concentrations of PCT have been observed compared to serum (Patel et al., 2015). Additionally, the antibody used in the assay and its possible affinity to different

conformations or states of PCT could lead to differences in immunoassays, as reported with other molecules such as oxytocin (MacLean et al., 2019). Moreover, PCT is a precursor of the Calcitonin Gene Family of Peptides, which means that other peptides very similar to PCT are also released into the bloodstream and could be easily detected by some assays. These peptides have different biological functions, but some share some similarities with PCT, like its possible increase in sepsis, such as adrenomedullin and calcitonin (Becker et al., 1981; Hu & Gagel, 2008).

According to these results, this assay could be potentially used as a tool for the non-invasive detection and monitoring of sepsis in pigs.

3.4.2. Comparison of different assays for the procalcitonin measurements in pigs (Experiment 2 in Annex)

- Aims and experimental design

This study aimed to develop monoclonal antibodies against porcine PCT, the first step for developing new assays that could improve sepsis diagnosis. Although polyclonal antibodies may recognize different epitopes and are easier to develop, monoclonal antibodies are highly specific and more stable for continuous production. In addition, different methods could detect the protein differently, which could help diagnose sepsis.

Currently, there is no commercial monoclonal antibody against porcine PCT, and This experiment aimed to develop and validate new assays with monoclonal antibodies for procalcitonin measurement in the saliva of pigs and compare their sensitivity and specificity with a previous polyclonal assay also validated in this PhD thesis.

The production of monoclonal antibodies was performed following the methods described in the section of general methods of this PhD thesis, based on the immunization of mice with recombinant pig procalcitonin, fusion of their spleen cells with myeloma, and hybridoma selection and cloning; in all cases using previously published protocols, more extensively described in the general methods section of this PhD thesis.

- Results and discussion

A total of 6 cell clones that produced monoclonal antibodies against pig PCT were obtained. The antibodies were purified with an affinity column and tested by ELISA and western blot methods, showing a high affinity to porcine PCT.

Currently, we are starting the development of the method using these monoclonal antibodies with the AlphaLISA technology used for other methods in this doctoral thesis, and soon the new assays will be validated following the procedures described in the general methods section. After that, a batch of saliva samples from pigs with different inflammatory and infectious diseases and from healthy pigs will be measured with the different obtained methods to compare their sensitivity and specificity to detect PCT in the samples with a different health status.

3.4.3. Validation of an assay for the measurement of presepsin in the saliva of pigs (Experiment 3 in Annex)

- Aims and experimental design

This study aimed to develop polyclonal and monoclonal antibodies with a high affinity to the protein presepsin (PSE, sCD14). PSE is closely related to sepsis because it appears when the receptor CD14 of the immune system has contact with bacterial antigens and cleaves itself. At present, no method exists to measure PSE in the pig; therefore, it is uncertain whether this biomarker could contribute to the diagnosis of sepsis.

For producing monoclonal and polyclonal antibodies, it was performed: 1) the immunization of a goat with PSE and the subsequent blood extractions to obtain serum with polyclonal antibodies, and 2) the immunization of mice with recombinant pig PSE, fusion of their spleen cells with myeloma, and hybridoma selection and cloning; in all cases using previously published protocols, more extensively described in the general methods section.

- Results and discussion

We obtained 2 cell clones that produced monoclonal antibodies against porcine PSE and a polyclonal antibody. All were tested by ELISA and western blot, displaying a high affinity to porcine PSE. The polyclonal and monoclonal antibodies were purified with an affinity column.

Developing these polyclonal and monoclonal anti-PSE antibodies will make it possible to test the combination of antibodies in different formats using AlphaLISA technology and to widen the number of assays to characterize sepsis in the pig. Once the methods are developed, the next step would be to validate them according to the procedures described in the general methods section of this PhD thesis. Ultimately, a

batch of saliva samples of pigs with inflammatory and infectious diseases and samples from healthy pigs will be measured with the resultant method to study the ability to detect sepsis in swine.

ARTICLES

Objective 1

Bibliographic research about the knowledge of sepsis and current most common biomarkers used to diagnose and monitoring this pathological state in veterinary medicine.

Article 1 (Published):

Biomarkers of sepsis in pigs, horses and cattle: from acute phase proteins to procalcitonin

Animal Health Research Reviews

cambridge.org/ahr

Biomarkers of sepsis in pigs, horses and cattle: from acute phase proteins to procalcitonin

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Review

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Abstract

Sepsis is a complex clinical syndrome triggered by an inflammatory host response to an infection. It is usually complicated to detect and diagnose, and has severe consequences in human and veterinary health, especially when treatment is not started early. Therefore, efforts to detect sepsis accurately are needed. In addition, its proper diagnosis could reduce the misuse of antibiotics, which is essential fighting against antimicrobial resistance. This case is a particular issue in farm animals, as antibiotics have been traditionally given massively, but now they are becoming increasingly restricted. When sepsis is suspected in animals, the most frequently used biomarkers are acute phase proteins such as C-reactive protein, serum amyloid A and haptoglobin, but their concentrations can increase in other inflammatory conditions. In human patients, the most promising biomarkers to detect sepsis are currently procalcitonin and presepsin, and there is a wide range of other biomarkers under study. However, there is little information on the application of these biomarkers in veterinary species. This review aims to describe the general concepts of sepsis and the current knowledge about the biomarkers of sepsis in pigs, horses, and cattle and to discuss possible advances in the field.

General concepts of sepsis

Sepsis, SIRS, and MODS

Sepsis is a complex life-threatening condition, being considered as one of the major causes of severe illness in veterinary species and human beings (Alberti *et al.*, 2003; Taylor, 2015). It occurs when an infectious agent triggers a systemic inflammatory response (SIRS) characterized by an imbalance between pro and anti-inflammatory elements that may cause disorders in hormonal, metabolic, cardiovascular, and coagulation systems, among others (Hotchkiss and Karl, 2003; Singer *et al.*, 2016; Smyth *et al.*, 2016). Therefore, sepsis has been defined as a SIRS caused by an infectious agent. In some cases, this inflammatory response can be deregulated and lead to multiple organ dysfunction syndrome (MODS), resulting in death (Levy *et al.*, 2003; Riedel and Carroll, 2013).

Sepsis has a significant impact on human health due to its severity and high mortality rates, being a global health priority by the World Health Organization and the World Health Assembly (WHO, 2017). In addition, sepsis and its consequences also significantly impact animal health. For example, calves and foals are highly vulnerable to endotoxemia, characterized by bacteremia or septicemia due to Gram-negative organisms, presenting very high mortality rates (Fecteau *et al.*, 2009; Bonelli *et al.*, 2015*a*, 2018). Therefore, sepsis is an example of a topic that should be addressed from the 'One-Health' point of view (Robinson *et al.*, 2016)

Causes

Although to the authors' knowledge there is no specific data in veterinary medicine, it was described that more than 70% of the documented sepsis cases in human beings are attributed to bacteria, leading to pneumonia and urinary tract, intra-abdominal, skin, and other soft tissue infections (Motzkus and Luckmann, 2017). Sepsis can also be triggered by other infectious agents like parasites, fungi, or viruses, although, for example, only around 1% of sepsis cases are classified as viral (Blanco *et al.*, 2008; Zahar *et al.*, 2011; Lin *et al.*, 2018). However, the prevalence of non-bacterial sepsis might be underdiagnosed because several reports indicated higher incidence. For example, one study reported that 21% of pediatric sepsis cases were attributed to viruses (Weiss *et al.*, 2015).

Physiopathology

The origin of sepsis relies on the interaction between the causal agent and the 'pattern-recognition receptors' (PRRs) present on cells of the innate immune response (Fig. 1).

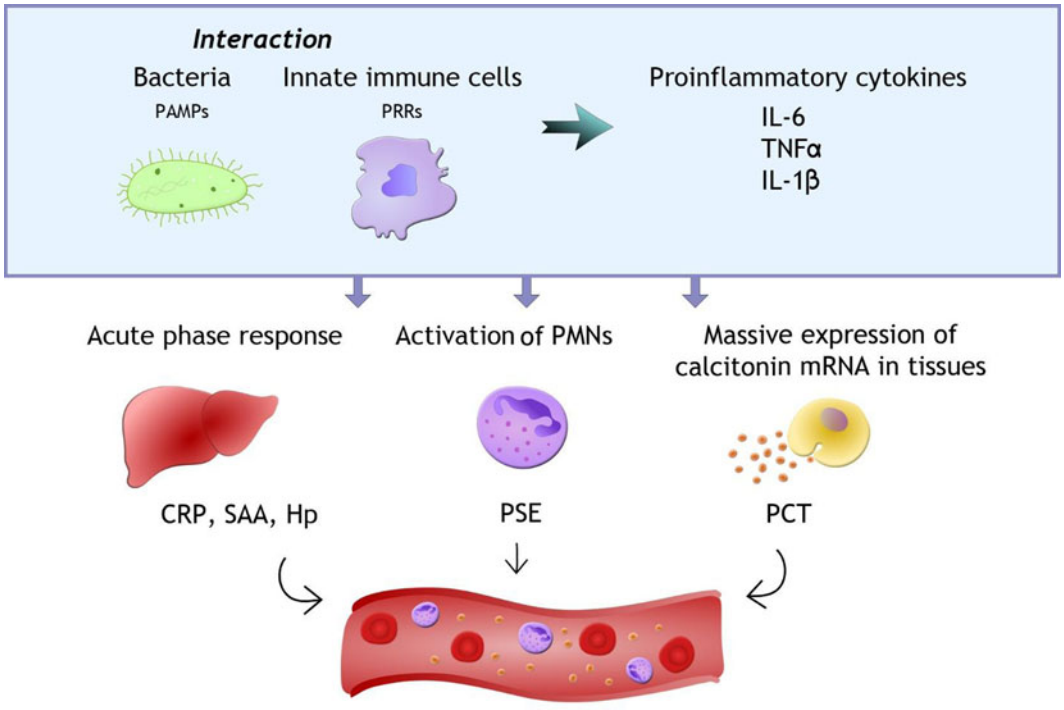

Fig. 1. Proinflammatory response during sepsis. PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; IL-6, interleukin-6; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β; CRP, C-reactive protein; SAA, serum amyloid A; PMN, polymorphonuclear leukocytes; PSE, presepsin; PCT, procalcitonin. Figure and images were created by the authors.

These receptors can detect the 'pathogen-associated molecular patterns' (PAMPs) that are conserved structures of pathogens. In addition, they can detect molecules produced by the host after damage, independent of the causal agent, known as 'damage-associated molecular patterns'. That is why sepsis and non-septic SIRS often have many similarities, making the diagnosis of sepsis challenging (Lewis *et al.*, 2012; Faix, 2013).

The interactions between the pathogen and cells trigger the production of proinflammatory cytokines, mainly interleukin-6 (IL)-6, tumor necrosis factor α (TNF-α), and IL-1β, that initiate the innate immune response and contribute to the onset of SIRS in two main ways, as they:

- Induce the acute-phase response with the production of acute phase proteins (APPs) such as C-reactive protein (CRP), serum amyloid A (SAA), or haptoglobin (Hp) (Faix, 2013).
- Activate endothelial cells and the production and attraction of polymorphonuclear leukocytes (PMNs) to the site of damage and into the general circulation. The activation of PMNs membrane receptors leads to the release of molecules such as sCD14 (known as presepsin (PSE)) or sTREM-1 from these receptors (Cray, 2012).

In addition, both the direct stimulation of the pathogens themselves, and the indirect stimulation by these three cytokines, induce the expression of calcitonin mRNA in numerous extrathyroidal tissues, leading to a widespread production of the protein procalcitonin (PCT) (Nakamura *et al.*, 2013).

This proinflammatory response evolved to eradicate the causative agent; however, if the response is too severe, organ damage and even organ dysfunction are likely to develop. Although some of the underlying mechanisms are still unknown, altered tissue oxygenation seems to be one of the main causes of the increase in severity. Several factors may be involved in this oxygenation imbalance, such as poor oxygen supply due to hypotension, cellular oxygen impairment due to mitochondrial damage from oxidative stress, and vascular endothelial dysfunction due to loss of barrier integrity and cell death. This endothelial damage and organ dysfunction lead to changes in angiopoietins or lactate, respectively (Angus and van der Poll, 2013; Faix, 2013).

Finally, another frequent complication of sepsis is the triggering of disseminated vascular coagulation. The link between inflammation and coagulation is complex, but it may be mediated primarily by protease-activated receptors type 1. Usually, these receptors have cytoprotective effects, but when stimulated by high doses of thrombin, their effects on the endothelial cell barrier become disruptive. Also, the imbalance of anticoagulant mechanisms and the suppression of the fibrinolytic system can lead to excessive fibrin deposition. Therefore, biomarkers of coagulation like D-dimer also have a role in monitoring sepsis (Angus and van der Poll, 2013).

It is important to point out that many of the mechanisms involved in sepsis remain unknown, especially the reasons for the variability of the response to infection. This variable response can depend on the causal agent, the host genetics, possible concomitant diseases, and animal species (Werling and Coffey, 2007; Lewis *et al.*, 2012; Gotts and Matthay, 2016).

Diagnosis

The diagnosis of sepsis is based on a confirmed infection and the presence of two or more SIRS criteria; although in some cases, the

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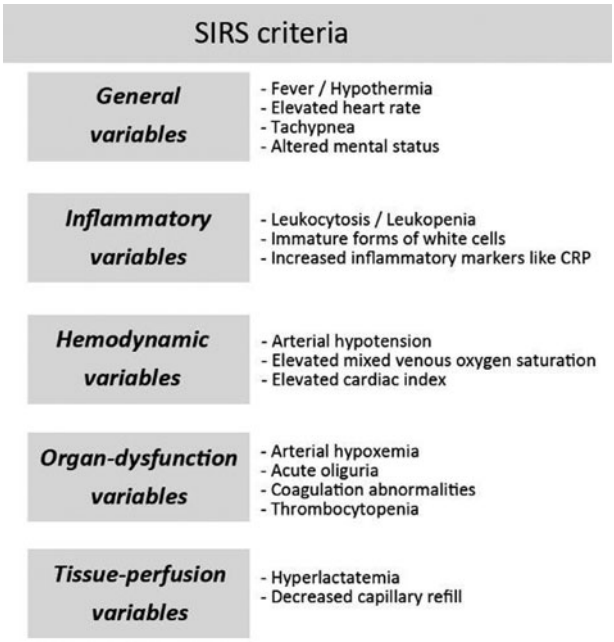

Fig. 2. Sepsis is suspected when the patient manifests two or more SIRS criteria with a possible infectious origin (Angus and van der Poll, 2013).

infection cannot be documented and is only suspected (Angus and van der Poll, 2013; Roy *et al.*, 2017). The SIRS criteria (Fig. 2) can vary, depending on the author, and generally include various external symptoms such as changes in body temperature, heart rate, or breathing pattern, as well as analytical abnormalities and signs for organ or tissue perfusion dysfunction (Fecteau *et al.*, 2009; Taylor, 2015; Peach, 2017). In human medicine, there are also score systems such as the sequential organ failure assessment score (SOFA), which evaluates breathing, circulation, liver and kidney function, coagulation, and neurologic condition. There is also a shortened version, the Quick-SOFA score, which just considers three points of the SOFA (Mccormack *et al.*, 2017; Serafim *et al.*, 2018; Garbero *et al.*, 2019). Some scores with adaptations have also been used in the veterinary field, such as the one modified by Corley and Furr (2003) that showed a low negative predictive value, or the sepsis score for horses adapted from Breuer and Schusser, which includes parameters such as calcium, fibrinogen, and body inner temperature (Breuer and Schusser, 2012).

Overall, several factors make the diagnosis of sepsis still a challenge:

- Initial clinical manifestations can be quite variable depending on individual factors such as the previous health status, the animal species, or the source of infection (Borghetti *et al.*, 2009; Motzkus and Luckmann, 2017).
- There are difficulties in many cases to detect the cause of sepsis. Although blood culture is the gold standard to detect bacteremia, it has many limitations, including a 48–72 h delay in results, a high rate of false negatives, and the possibility of false positives by sample contamination (Shozushima *et al.*, 2011; Singer *et al.*, 2016; Jereb *et al.*, 2019). In addition, there is a lack of diagnostic criteria for other causal agents such as viruses, probably resulting in underestimation of the incidence of non-bacterial sepsis (Amin and Amin, 2015; Gotts and Matthay, 2016).
- Inflammation and SIRS without any infectious agent involved can lead to very similar clinical features (Pierrakos and Vincent, 2010).

These challenges can limit the correct diagnosis of sepsis, and thus the initiation of a rapid and adequate treatment to ensure that the patient's life is not endangered. Sepsis treatment is complex and can require intensive care to provide vital-organ support. When the origin is bacterial, the critical element is the rapid inclusion of antibiotics, as delays in antimicrobial therapy can significantly increase the risk of dying (Martin, 2012). Therefore, antibiotic treatment is frequently administered in a preventive approach without the confirmation of the bacterial component. This fact results in excessive and sometimes unnecessary use of antibiotics, which increase medical costs, are harmful to host microbiota, and contribute to the development of multidrug-resistant bacteria (Lin *et al.*, 2018; Tosi *et al.*, 2018; Grondman *et al.*, 2020). Antibiotic resistance is increasingly leading to treatment failures and worse prognoses in both infectious and non-infectious conditions (Peralta *et al.*, 2007; Hocking *et al.*, 2021). This overuse of antibiotics is widespread in farm animals, particularly serious in porcine production and remains a crucial area for improvement (Van Boeckel *et al.*, 2015; Murphy *et al.*, 2017).

Roles of biomarkers in sepsis

The use of biomarkers for the early diagnosis and monitoring of sepsis can help to improve its management and treatment. Although more than 170 potential biomarkers have been described in human medicine for assessing the diagnosis and prognosis of sepsis, there is still a lack of consensus about their actual usefulness in practice (Pierrakos and Vincent, 2010; Ivady *et al.*, 2011; Reinhart *et al.*, 2012; Liu *et al.*, 2016).

In veterinary medicine, it is also necessary to improve both the diagnosis and prognosis of sepsis. Traditionally, the most used biomarkers to detect sepsis in animals are APPs such as CRP, as well as proinflammatory cytokines. However, these biomarkers have limitations: APPs have low specificity to detect sepsis because they increase in many non-septic conditions, while cytokines, in addition to their low specificity, have a short half-life that makes their detection difficult (Ercan *et al.*, 2016). New sepsis biomarkers are emerging on the veterinary side, with the intent to overcome these drawbacks (Ercan *et al.*, 2016; Kirbas *et al.*, 2019).

Lipopolysaccharide (LPS) endotoxin concentrations have been measured with ELISA kits and reported as potential biomarkers. However, LPS molecules can decrease significantly within one hour after taking the sample, and the interpretation of the results is quite complex, as they are heterogeneous and highly variable depending on the bacteria from which they originated. In addition, LPS molecules detected in plasma could be a tiny proportion of LPS remaining after clearance of bacterial infections and would no longer represent the initial amounts of LPS capable of stimulating host cells. Therefore, LPS concentrations have limited clinical utility (Peek *et al.*, 2004; Senior *et al.*, 2011; Gnauck *et al.*, 2016).

This review aims to update the state of the main biomarkers currently used to diagnose sepsis in large farm animals (namely pigs, horses, and cattle) and other biomarkers that could potentially be applied in veterinary medicine. For this purpose, they will be classified here into traditional biomarkers of sepsis,

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specific biomarkers for bacterial sepsis, and biomarkers that can provide additional information in sepsis.

Traditional biomarkers of sepsis

This category includes APPs and cytokines.

Acute phase proteins (APPs)

The acute phase response is part of the first-line host defense in response to pathogenic states such as infection, inflammation, or tissue damage. In response to these stimuli, first-line defense cells activate the release of cytokines that induce changes in the concentration of APPs. APPs are helpful to diagnose and monitor even subclinical conditions that can lead to impaired growth and reproductive performances in animals (Petersen *et al.*, 2004; Quereda *et al.*, 2012; Schrödl *et al.*, 2016).

The origin of most APPs has been traditionally associated with the liver, although there is growing evidence that it can also be extra-hepatic. According to their dynamics, APPs can be classified as positive if their concentrations increase in the acute phase reaction or as negative if their concentrations decrease. Positive APPs can also be classified as major (with very high and rapid increases upon stimulation and rapid decreases after cessation of the stimulus) and moderate (with milder and slower dynamics in general). The classification and pattern of the response differs among animal species (Eckersall and Bell, 2010; Cerón *et al.*, 2014).

The main advantage of APPs is that their basal levels are very low compared to other biomarkers of inflammatory responses, and, generally, marked increases in major APPs together with compatible external clinical signs can lead to the possible diagnosis of sepsis. However, rises of high magnitude can also occur in other inflammatory conditions, especially in immune-mediated pathologies, and APPs generally show a low specificity to detect bacterial sepsis (Eckersall and Bell, 2010; Reczyńska *et al.*, 2018). Nevertheless, because APP assays are becoming more available and affordable, they can provide valuable information in the daily farm routine, mainly in complementing the diagnosis, monitoring the development of the disease, and establishing the patient's prognosis (Cerón, 2019).

C-reactive protein (CRP)

This positive APP belongs to the pentraxin family and is one of the most traditionally used biomarkers in diagnosing sepsis in people and animals (Ansar and Ghosh, 2013; Enguix-Armada *et al.*, 2016). Its primary biologic functions include complement activation, opsonization, and modulation of monocytes and macrophages and cytokine production (Ballou and Lozanski, 1992; Singh *et al.*, 2020). In species in which it is a major APP, such as the pig, serum CRP concentration can increase greater than 100-fold; normally, it rises above physiological values as soon as 6 h after the stimulus and has a half-life of about 47 h (Jain *et al.*, 2011; Takata *et al.*, 2011). CRP measurement allows a fast and economical evaluation of the inflammatory response at an early stage, which can provide information about the severity of the pathology and the response to the treatment (Flanders *et al.*, 2004; Schmit and Vincent, 2008). However, its role in diagnosing sepsis is limited by its variable sensitivity and specificity, which, respectively, have been reported to be 30–97.2% and 75–100% among human subjects (Wyllie *et al.*, 2005; Chan and Gu, 2011). Therefore, in human medicine, CRP is being replaced or combined with other markers such as PCT (Müller *et al.*, 2007; Qu and Summah, 2009; Kibe *et al.*, 2011).

Examples of studies involving CRP in large animals have been summarized in Table 1, and are tabulated according to species, pathological condition (bacterial infections; non-bacterial infections – viral, parasitic, or fungal-; and non-septic inflammation), and analytical method used for CRP measurement. The interpretation of CRP is different for pigs, horses, and ruminants. In pigs, it is considered a major APP: CRP concentration rises in many inflammatory diseases, including those caused by bacterial infections (Lauritzen *et al.*, 2003; Soni and Adebiyi, 2017; Yin *et al.*, 2017), viral infections, and non-infectious etiologies (Gómez-Laguna *et al.*, 2010; Escribano *et al.*, 2015*a*). Therefore, its usefulness to discern bacterial sepsis is unclear (Heegaard *et al.*, 2011). In horses, CRP is considered a moderate APP that slightly increases 3–5 days after stimuli, and does not appear useful to detect sepsis because it increases similarly to non-bacterial inflammation (Yamashita *et al.*, 1991; Do Carmo *et al.*, 2015; Taylor, 2015; Zabrecky *et al.*, 2015). In cattle, CRP is generally not considered an APP, as no acute increases have been described in certain pathologies such as respiratory disease (Nakajima *et al.*, 1993; Prohl *et al.*, 2015), although some studies have shown mild CRP increases under conditions such as stress, intestinal obstruction, clinical mastitis, parasitic infections, or septicemia, which could make CRP useful in assessing the general status of herds (Lee *et al.*, 2003; Hussain *et al.*, 2015; Akgül *et al.*, 2019; Appelt *et al.*, 2019; Dalanezi *et al.*, 2020). Currently, because its measurement costs are similar to other routinely-measured methods, CRP could be measured on a routine basis to increase sensitivity to detect inflammation, especially in pigs (Tecles *et al.*, 2007; Rivera-Gomis *et al.*, 2020).

Serum amyloid A (SAA)

This term groups a family of polymorphic apolipoproteins with a half-life of around 35 h (Çetinkaya *et al.*, 2009; Takata *et al.*, 2011). SAA has several biologic functions including a chemotactic effect on monocytes, PMNs, and T cells, an inhibitory effect on IL-1, and TNF-induced fever (Shainkin-Kestenbaum *et al.*, 1991; Badolato *et al.*, 1994). Its main use in human sepsis has been for detecting neonatal sepsis, with a sensitivity ranging from 23 to 100% and a specificity ranging from 44 to 100% (Yuan *et al.*, 2013).

Studies of SAA in large animals have been summarized in Table 2, which are classified according to species, pathological condition (bacterial infections; non-bacterial infections – viral, parasitic, or fungal-; and non-septic inflammation), and analytical method used for SAA measurement. In veterinary medicine, SAA is considered the major APP in horses and one of the major APPs in cattle and pigs (Hulten and Demmers, 2002; Pradeep, 2014). Its concentration rises in experimental and natural infections of different natures (Campbell *et al.*, 2005; Kabu *et al.*, 2016; Joshi *et al.*, 2018), including viral infections (Heegaard *et al.*, 2000; Sánchez-cordón *et al.*, 2007) and other conditions such as local aseptic inflammation, traumatic reticuloperitonitis, non-infectious diarrhea, or surgery (Nunokawa *et al.*, 1993; Jacobson *et al.*, 2001; Parra *et al.*, 2006; Pourjafar *et al.*, 2011; Baydar and Dabak, 2014). In some of these cases, SAA can remain high for some time after the trigger has disappeared (Aitken *et al.*, 2019). The existence of immunoturbidimetric assays that can be adapted to automated analyzers allows the use of this APP in routine diagnostics (Franco-Martínez *et al.*, 2021).

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Table 1. Examples of research about C-reactive protein (CRP) in farm animals with different causal agents

Animal	Causal agent		Disease	Analytical method	Subjects	Values[a]		Ref.
						Control group[b]	Diseased group	
Pig	*Infectious*	*Bacterial*	Induced polymicrobial sepsis by cecal ligation procedure (CLP)	Commercial ELISA kit (Immunology Consultants Laboratory, Portland OR)	7 neonatal pigs (+control group: 6) Age: 3–5 days	Approx.[c] 40 mg l^{-1} (Sham-operated without CLP)	Approx.[c] 100 mg l^{-1}	Soni and Adebiyi (2017)
			Experimental infection with *Streptococcus suis*	In-house porcine ELISA	5 pigs Age: 8 weeks	5.13 mg l^{-1} (time 0, pre-inoculation)	Approx.[c] 60–90 mg l^{-1} (graph)	Sorensen *et al.* (2006)
		Non-bacterial	Induced experimental infection with *PRRSV*	Commercial ELISA kit (PhaseTM; Tridelta Development Ltd, Ireland)	28 pigs (+control group: 4) Age: 5 weeks	10 mg l^{-1}	Max. values at day 17 post inoculation (2.55-fold increase)	Gómez-Laguna *et al.* (2010)
	Non-infectious[d]		Experimental inflammation with turpentine	Immunoturbidimetric human assay previously validated in pigs	5 pigs (45 kg) (+control group: 5)	<25 mg l^{-1}	Max. concentration: 162 mg l^{-1}	Escribano *et al.* (2015*b*)
Horse	*Infectious*	*Non-bacterial*	3 seropositive groups for *Toxoplasma gondii*	Commercial kit of ultrasensitive CRP (BioTécnica, Brazil)	45 adult horses (+control group: 20)	0.45 mg dl^{-1}	2.0, 16.2, and 38.3 mg dl^{-1} per group	Do Carmo *et al.* (2015)
		Bacterial	Sepsis	Commercial ELISA kit (Horse CRP ELISA, Kamiya Biomedical Company, Seattle, WA)	80 foals (40 foals each group) Age: <1 week	0–240 mg ml^{-1} (range)	0–336 mg ml^{-1} (range)	Zabrecky *et al.* (2015)
	Non-infectious[d]		Diseases with a negative sepsis score				0–260 mg ml^{-1} (range)	
			Induced experimental inflammation (turpentine)	Single radial immunodiffusion assay with in-house polyclonal antibodies	3 horses Age: 3–12 years	<10 mg l^{-1} (time 0, pre-inoculation)	Approx.[c] 30 mg l^{-1}	Yamashita *et al.* (1991)
			Castration		5 horses Age: 4–7 years		Approx.[c] 30 mg l^{-1}	
Cattle	*Infectious*	*Bacterial*	Presumed septicemia	Commercial ELISA device (DAS, Italy)	20 neonatal calves (+control group: 10) Age: 1–10 days	13.64 ng l^{-1}	24.19 ng l^{-1}	Akgül *et al.* (2019)
		Non-bacterial	Seropositive for *Neoplasma caninum*	Ultrasensitive CRP commercial kit (BioTécnica, Minas Gerais, Brazil) on a Bio-2000 analyzer (BioTécnica, Minas Gerais, Brazil)	86 seropositive cows (+control group: 176) Age: 2–8 years	6.57 mg dl^{-1}	11.72 mg dl^{-1}	Appelt *et al.* (2019)
	Non-infectious[d]		Intestinal obstruction	Bayer's diagnostic kit with Microlab Auto-analyser (Merck)	6 cows, 2 buffaloes (+control group: 10 and 10)	0.23 mg dl^{-1}	4.16 mg dl^{-1}	Hussain *et al.* (2015)

[a]Values: results expressed as the arithmetic means of the values of the group unless otherwise stated.
[b]Control group: animals classified as healthy, unless specified that the selection procedure for the control group is different.
[c]Approximately (based on the graph presented in the referenced article).
[d]Non-infectious disease: this category includes pathologies classified as non-infectious but not necessarily confirmed to be free of secondary infectious agents.

Table 2. Examples of research about serum amyloid A (SAA) in farm animals with different causal agents

Animal	Causal agent		Disease	Analytical method	Subjects	Values[a]		Ref.
						Control group[b]	Diseased group	
Pig	*Infectious*	*Bacterial*	Experimental infection with *Streptococcus suis*	Commercial ELISA kit (PhaseTM; Tridelta Development Ltd, Ireland)	5 pigs Age: 6 weeks	Undetectable (pre-inoculation)	232 mg l^{-1}	Campbell *et al.* (2005)
		Non-bacterial	Experimental infection with classical swine fever (CSF) and African swine fever (ASF) virus	Commercial ELISA kit (PhaseTM; Tridelta Development Ltd, Ireland)	2 groups of 4 pigs Age: 10 weeks	1.14 mg l^{-1} in CSF and 2.75 mg l^{-1} in ASF group (pre-inoculation)	Max. concentrations: 359.9 in CSFv and 112.18 mg l^{-1} in ASFv	Sánchez-cordón *et al.* (2007)
	Non-infectious[c]		Bites, arthritis, rectal prolapse, ulcerated umbilical hernia	Commercial ELISA kit (PhaseTM; Tridelta Development Ltd Ireland)	16 pigs (+control group: 17) Age: 15 weeks	3.10 mg l^{-1} (median)	26.05 mg l^{-1} (median)	Parra *et al.* (2006)
Horse	*Infectious*	*Bacterial*	Neonatal weakness, pneumonia, and diarrhea with confirmed bacterial infections	Non-competitive enzyme immunoassay validated for horses	25 foals Age: 1 day to 3 months	1.6 mg l^{-1} (median) (non-bacterial diagnoses)	65 mg l^{-1} (median)	Hulten and Demmers (2002)
			Natural infection by *Streptococcus equi*	Stall-side lateral flow immunoassay test	44 adult horses	Undetectable	1953 mg l^{-1} in *S. equi*, 731 mg l^{-1} in EIV and 1173 mg l^{-1} in EHV-4 (median)	Viner *et al.* (2017)
		Non-bacterial	Natural infection by equine influenza virus (EIV) and equine herpesvirus-4 (EHV-4)		Adult horses, 42 infected by EIV and 43 by EHV4 (+control group: 40)			
	Non-infectious[c]		Surgery after colic	Commercial point-of-care quantitative lateral flow immunoassay	51 horses Age: >2 years (median: 11 years)	Undetectable (at admission)	Max. concentration: 989 mg l^{-1} without complications; 1664 mg l^{-1} with complications	Aitken *et al.* (2019)
Cattle	*Infectious*	*Bacterial*	Endotoxemia induced by *Lipopolysaccharide* inoculation	Commercial ELISA kit (Invitrogen Corporation, Carlsbad, USA)	8 calves Age: 25–42 days	12.4 mg l^{-1} (pre-inoculation)	Max. concentration: 185 mg l^{-1}	Coskun and Sen (2012)
			Naturally bovine respiratory disease (BRD) with isolated bacteria	Commercial ELISA kit (Sincere Biotech Co. Ltd)	12 calves (+control group: 12) Age: 2 weeks to 6 months	304 ng ml^{-1}	Max. concentration: 979.74 ng ml^{-1}	Joshi *et al.* (2018)
		Non-bacterial	Experimental infection with bovine respiratory syncytial virus	Commercial ELISA kit (PhaseTM; Tridelta Development Ltd, Ireland)	6 calves (+control group: 4) Age: 7–14 days	<17 mg l^{-1}	60–80 mg l^{-1} (range)	Heegaard *et al.* (2000)
	Non-infectious[c]		Acute traumatic reticuloperitonitis	Commercial ELISA kit (PhaseTM; Tridelta Development Ltd, Ireland)	10 cows (+control group: 10) Age: 3–9 years	18.50 mg l^{-1}	110.80 mg l^{-1}	Baydar and Dabak (2014)

[a]Values: results expressed as the arithmetic means of the values of the group unless otherwise stated.
[b]Control group: animals classified as healthy, unless specified that the selection procedure for the control group is different.
[c]Non-infectious disease: this category includes pathologies classified as non-infectious but not necessarily confirmed to be free of secondary infectious agents.

Haptoglobin (Hp)

Hp is an α2-globulin that reduces the availability of the heme residue by binding free hemoglobin released from erythrocytes, which prevents oxidative damage to tissues and has indirect bacteriostatic and immunomodulatory effects (Petersen *et al.*, 2004; Tthov *et al.*, 2013). Hp increases moderately in experimental and natural infections, including septicemia, and has a half-life of 3–5 days (Jain *et al.*, 2011; Coskun and Sen, 2012; Joshi *et al.*, 2018; Kirbas *et al.*, 2019). In human beings, a sensitivity of 67% and a specificity of 95% in the detection of neonatal sepsis has been reported (Emami *et al.*, 2016). However, this biomarker has been studied less than other biomarkers in sepsis.

Examples of studies of Hp in large animals have been summarized in Table 3, classifying them according to species, pathological condition (bacterial infections; non-bacterial infections – viral, parasitic, or fungal-; and non-septic inflammation), and analytical method used for Hp measurement. Hp is a major APP in cattle (Eckersall and Bell, 2010), and in combination with SAA, Hp can potentially be used in the diagnosis and the distinction between acute and chronic inflammation (Alsemgeest *et al.*, 1994; Horadagoda *et al.*, 1999). In the pig, Hp is a moderate APP and has been included in the optimal combinations of APPs to detect infectious diseases of swine (Heegaard *et al.*, 2011; Pradeep, 2014). In horses, Hp also is a moderate APP; thus, its increases are usually lower and delayed, and its concentration can remain high even after the cause has been resolved (Jacobsen and Andersen, 2007; Crisman *et al.*, 2008). Among these three species, Hp concentration can also rise in non-infectious pathologies such as local aseptic inflammation, fatty liver, and after transport or surgeries (Murata and Miyamoto, 1993; Nakagawa *et al.*, 1997; Parra *et al.*, 2006; Guzelbektes *et al.*, 2010; Heinonen *et al.*, 2010; Zabrecky *et al.*, 2015), as well as in infectious pathologies caused by viruses and parasites (Heegaard *et al.*, 2000; Pomorska-Mól *et al.*, 2014; El-Deeb *et al.*, 2018). In addition to immunoturbidimetric methods, the existence of spectrophotometric assays that can be automated allows the use of Hp in routine diagnostics (Brady *et al.*, 2019). Hp as well as other APPs can also be measured in saliva (Cerón *et al.*, 2019).

Other APPs

There are other positive APPs that could also have potential in the diagnostic and monitoring of sepsis, such as alpha 1-acid glycoprotein or porcine major acute phase protein in pigs (Moore *et al.*, 1997; Martín de la Fuente *et al.*, 2010). In addition, there are negative APPs such as albumin, apolipoprotein-1, and paraoxonase-1, which decrease in farm animals in the acute-phase response, that could be also useful in the diagnosis, prognosis, and monitoring response to treatment of sepsis (Heegaard *et al.*, 2011; Schneider *et al.*, 2013; Escribano *et al.*, 2015*b*; Scavone *et al.*, 2020).

Cytokines and chemokines

Cytokines and chemokines are a large group of proteins that include those released during the innate immune response as initial mediators in the inflammatory reaction. They regulate the nature of the immune response produced after a stimulus and are essential in cell activation and recruitment, antigen presentation, cell differentiation in the bone marrow, and in general development and modulation of the inflammatory reaction. The main difference between chemokines and cytokines is that chemokines can induce chemotaxis of cells such as neutrophils. Most cytokines and chemokines are produced very early in sepsis and have a short half-life of around 4 h (Borish and Steinke, 2003; Orlikowsky *et al.*, 2004; Monastero and Pentyala, 2017).

Two of the most frequently studied cytokines in human beings and animals are TNF-α and IL-6. Both have been observed to increase in pigs and cattle, mainly under experimental conditions, like induced endotoxemia and bacterial infections (Basoglu *et al.*, 2004; Castellheim *et al.*, 2008; Ballou *et al.*, 2011; Wyns *et al.*, 2015).

Cytokines could be helpful to evaluate the severity of sepsis and to help determine etiology. For example, plasma IL-6 increases more in bacterial infections, and IL-8 or IL-18 concentrations are higher when the causal agent is a Gram-negative or Gram-positive bacteria, respectively (Feezor *et al.*, 2003; Holub *et al.*, 2013; Surbatovic *et al.*, 2015). However, the short half-lives of most cytokines can lead to false-negative results, and there is often a high degree of inter-individual variability and low specificity, which makes these tests difficult to evaluate (Arnon and Litmanovitz, 2008; Gentile *et al.*, 2014). Some authors have proposed that the development of diagnostic test panels with cytokine combinations could be beneficial in characterizing the severity of the disease (Kabir *et al.*, 2003; Punyadeera *et al.*, 2010; Mera *et al.*, 2011). However, the limitations of short target half-lives, low diagnostic specificity, and a dearth of automated and affordable assays in the veterinary field restrict the use of such tests mainly to research (Scheerlinck and Yen, 2005).

Specific biomarkers of bacterial sepsis

PCT and PSE are increasingly used in diagnosis of human sepsis, due to their higher specificity for detecting infectious disease, with strong scientific evidence of their usefulness as part of the diagnosis of sepsis (Yang *et al.*, 2014; Wu *et al.*, 2015). In addition to PCT and PSE, in this section we will describe other biomarkers that can have potential to diagnose sepsis.

Procalcitonin (PCT)

PCT is one of the most widespread biomarkers of sepsis in human medicine (Schneider and Lam, 2007). Normally, this glycoprotein is mainly produced in the thyroid C cells and pulmonary-endocrine cells, and is the precursor of calcitonin, a hormone with a metabolic role in calcium homeostasis (Becker *et al.*, 2010). In healthy individuals, almost all PCT is converted into mature calcitonin; therefore, PCT concentrations in serum from healthy human subjects are low (Nakamura *et al.*, 2013). In some pathologic states like sepsis, proinflammatory mediators induce massive calcitonin mRNA expression, and extra-thyroidal PCT is produced in many tissues. As there are no secretory granules in these tissues, PCT is released into the bloodstream without being converted to calcitonin (Morgenthaler *et al.*, 2003; Matur *et al.*, 2017). In these cases, its concentration can rise to thousands-fold with a half-life of 25–30 h, starting to increase after 3 h and reaching maximum levels at 6 h (Nijsten *et al.*, 2000; Carrol *et al.*, 2002). The degree of increase of PCT correlates with the severity and etiology of the pathology, with mild increases in local infections (Assicot *et al.*, 1993; Giamarellos-Bourboulis *et al.*, 2001).

Initial studies hypothesized that the massive production of PCT could be related to the hypocalcemia that frequently appears in patients with sepsis. However, this hypothesis was not

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Table 3. Examples of research about haptoglobin (Hp) in farm animals with different causal agents

Animal	Causal agent		Disease	Analytical method	Subjects	Values[a]		Ref.
						Control group[b]	Diseased group	
Pig	*Infectious*	*Bacterial*	Endotoxemia induced by *Lipopolysaccharide* inoculation	Commercial ELISA kit (Shangai Kexing Trading Co., China)	6 male piglets (12 kg) (+control group: 6)	Max. concentration <60 μg l^{-1} (graph)	Max. concentration >60 μg l^{-1} (graph)	Yin *et al.* (2017)
		Non-bacterial	Naturally infected with Porcine Circovirus Type 2 (PCV2)	Commercial ELISA kit (Phase Range; Tridelta Development Ltd Ireland)	10 animals (+control group: 17) Age: 16 weeks	0.21 g l^{-1} (median)	5.03 g l^{-1} (median)	Parra *et al.* (2006)
			Inoculation of H1N1 swine influenza virus	Commercial ELISA kit (Life Diagnostics, Inc., USA)	9 pigs (+control group: 5) Age: 6 weeks	<0.65 g l^{-1} (Mock-infected with PBS)	Maximum individual concentration: 3.37 g l^{-1}	Pomorska-Mól *et al.* (2014)
	Non-infectious[c]		Pigs with bitten tails	Hemoglobin-binding assay for bovines with modifications	12 piglets (+control group: 13)	1.2 g l^{-1} (median)	2.8 g l^{-1} (median)	Heinonen *et al.* (2010)
Horse	*Infectious*	*Bacterial*	Severe sepsis	Commercial ELISA kit (Kamiya Biomedical Company, Seattle, WA, USA)	40 septic foals (+control group: 39) Age: <1 week	1627 g l^{-1}	1190 g l^{-1} (septic foals)	Zabrecky *et al.* (2015)
		Non-bacterial	Clinical cutaneous habronemosis	Commercial ELISA kit (Phase kit, Tridelta Ltd., Ireland)	30 foals (+control group: 20)	0.74 g l^{-1}	1.61 g l^{-1}	El-Deeb *et al.* (2018)
	Non-infectious[c]		Inflammatory airway disease (IAD)	Commercial ELISA kit (Cederlane, Burlington, ON, Canada)	12 horses (+control group: 10) Age: 6–14 years	1.18×10^6 ng ml^{-1}	Max. concentration: 10.6 × 10^6 ng ml^{-1}	Bullone *et al.* (2015)
Cattle	*Infectious*	*Bacterial*	Endotoxemia induced by *Lipopolysaccharide* inoculation	Commercial ELISA kit (Invitrogen Corporation, CA, USA)	8 calves Age: 25–42 days	8.13 μg ml^{-1} (preinoculation)	Max. concentration: 500 μg ml^{-1}	Coskun and Sen (2012)
		Non-bacterial	Experimental infection with bovine respiratory syncytial virus	Commercial ELISA kit (Phase kit, Tridelta Ltd., Ireland)	6 calves (+control group: 4) Age: 7–14 days	Below the detection limit of the ELISA	Max. concentration: 10 g l^{-1}	Heegaard *et al.* (2000)
	Non-infectious[c]		Left (LDA) and right (RDA) displacement of the abomasum with fatty liver	Commercial ELISA kit (Life Diagnostics Inc, West Chester, PA, USA)	42 cows with LDA and 16 with RDA (+control group: 9) Age: 3–8 years	36 μg ml^{-1}	70 μg ml^{-1} in LDA and 88 μg ml^{-1} in RDA	Guzelbektes *et al.* (2010)

[a]Values: results expressed as the arithmetic means of the values of the group unless otherwise stated.
[b]Control group: animals classified as healthy, unless specified that the selection procedure for the control group is different.
[c]Non-infectious disease: this category includes pathologies classified as non-infectious but not necessarily confirmed to be free of secondary infectious agents.

supported because levels of mature calcitonin usually did not increase during sepsis, and it is currently hypothesized that the cause of hypocalcemia may be multifactorial (Becker *et al.*, 2004; Holowaychuk and Martin, 2007). Overall, the presence of hypocalcemia does not seem to be a specific biomarker of sepsis because it can be produced by other causes such as hypoparathyroidism, pseudohypoparathyroidism, hypomagnesemia, hyperphosphatemia, pancreatitis, or vitamin D deficiency, which can also be triggered by some secondary liver or intestinal problems (Kelly and Levine, 2013).

To date, the role of PCT in sepsis is not clear. There are studies suggesting a proinflammatory effect by increasing the expression of surface markers on neutrophils and lymphocytes or by increasing the concentration of intracellular calcium ions (Wei *et al.*, 2008), while other studies have reported a possible anti-inflammatory role (Monneret *et al.*, 2000; Matera *et al.*, 2012). The intraperitoneal administration of exogenous PCT to septic hamsters was toxic and increased their mortality, whereas the same administration in healthy animals did not have any effect (Becker *et al.*, 2010). Therefore, deeper research in PCT functionality is necessary to better understand this biomarker.

As a biomarker of sepsis, the main advantages of PCT are the longer half-life and stability in blood than cytokines, and the higher specificity than APPs and cytokines for detecting responses to bacterial infections. Regarding specificity, in viral infections, the main mediator is Interferon-γ (IFN-γ), which inhibits transcription of PCT mRNA (Linscheid *et al.*, 2003). Increased PCT concentrations have been observed in some non-infectious inflammatory conditions such as severe burns, trauma, or major surgery (Becze, 2016). However, these increases in PCT concentration after surgery are usually moderate and generally last less than 48 h, contrarily to infectious processes, where the increments are much higher. Overall, the diagnostic accuracy of PCT has been reported among 74.8–100% of sensitivity and 70–100% of specificity, and in some studies was able to distinguish between bacterial and non-bacterial infection better than blood culture (Aikawa *et al.*, 2005; Chan and Gu, 2011). PCT facilitates an earlier diagnosis of sepsis and correlates well with the risk of mortality (Jensen *et al.*, 2006; Liu *et al.*, 2015; Hamo *et al.*, 2017). In addition, PCT can guide clinicians to provide better antibiotic treatment to minimize the overuse of these drugs without compromising the safety of patients (Mehanic and Baljic, 2013; Sager *et al.*, 2017). The most commonly used PCT ranges for clinical decisions with respect to sepsis in humans are exemplified in Table 4 (Schuetz *et al.*, 2007, 2011; Sager *et al.*, 2017).

Studies about PCT in large animals have been summarized in Table 5, classifying them according to species, pathological condition (bacterial infections; non-bacterial infections – viral, parasitic, or fungal-; and non-septic inflammation), and analytical method used for PCT measurement. Compared to APPs, the number of studies on PCT in animals is relatively low. In the horse, basal levels seem to be higher than in human beings (Rieger *et al.*, 2014; Bonelli *et al.*, 2017). This difference could be due to interspecific differences, such as the close contact with the large amounts of LPS that are generally in the equine intestinal lumen (Dicks *et al.*, 2014). Increases in PCT concentrations have been observed in horses with natural sepsis, after induced endotoxemia and enterocolitis, and in colic (Bonelli *et al.*, 2015*a*, 2015*b*; Teschner *et al.*, 2015; El-Deeb *et al.*, 2020). Regarding cattle, increases in PCT concentrations have been observed in systemic colibacillosis, newborn calves with sepsis, and in cows with a left abomasum displacement. In healthy calves, the concentrations were similar to those found in horses (Ercan *et al.*, 2016; Bonelli *et al.*, 2018; Kirbas *et al.*, 2019). In pigs, only a few studies with induced experimental infections found measurable concentrations of plasmatic PCT, maybe due to the lack of specificity of the methods (Wagner *et al.*, 2002; Zannoni *et al.*, 2012; Spyropoulos *et al.*, 2019; Liu *et al.*, 2021). To the authors' knowledge, very few studies have evaluated PCT in non-septic inflammation (Ismail *et al.*, 2019; Kilcoyne *et al.*, 2020), and there are no studies about the role of PCT during viral pathologies in animals.

Table 4. Procalcitonin algorithm used in critically ill human patients with suspected sepsis (Schuetz *et al.*, 2007, 2011; Sager *et al.*, 2017)

PCT (ng ml^{-1})	Odds of sepsis	Antibiotic treatment recommendations
<0.1	Very improbable	• At admission: antibiotics not recommended • After treatment: discontinue antibiotics
0.1–0.25	Very improbable	• At admission: consider initial empiric antibiotic treatment • After treatment: consider discontinue antibiotics
0.25–0.5	Improbable	• At admission: antibiotic treatment recommended • After treatment: consider discontinue antibiotics with close clinical evaluation
0.5–1	Probable	• At admission: antibiotics are needed • After treatment: daily measurement of PCT until levels decrease >80%
>1	Very probable	
>10	Suggestive of septic shock	

Presepsin (PSE)/Soluble cluster of differentiation 14 subtype (sCD14-ST)

PSE is a soluble product that comes from the cleavage of the CD14 glycoprotein. These glycoproteins are located mostly on PMN surfaces and act as receptors that can bind to bacterial structures, which can result in their proteolysis, leading to the release of sCD14 into the blood (Chenevier-Gobeaux *et al.*, 2015; Memar and Baghi, 2019).

In human beings, PSE can be useful in the diagnosis and prognosis of local infections and in septic SIRS (Endo et al., 2012), compared to non-infectious SIRS (Shozushima *et al.*, 2011; Endo *et al.*, 2012; Behnes *et al.*, 2014). Some studies have concluded that PSE showed better performance in diagnosing sepsis than PCT, but other studies have not confirmed such superiority (Yaegashi *et al.*, 2005; Liu *et al.*, 2013; Romualdo *et al.*, 2014; Zhu *et al.*, 2020). Overall, the sensitivity and specificity of PSE have been reported as 76–80% and 80–85%, respectively.

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Table 5. Examples of research about procalcitonin (PCT) in farm animals with different causal agents

Animal	Causal agent		Disease	Analytical method	Subjects	Values[a]		Ref.
						Control group[b]	Diseased group	
Pig	*Infectious*	*Bacterial*	Experimental model of rapidly lethal polymicrobial peritonitis	Radio-immunoassay (RIA) with rabbit antiporcine N-ProCT antiserum	1 pig (+control group: 1)	<50 fmol ml^{-1}	Max. concentration: 375 fmol ml^{-1}	Wagner *et al.* (2002)
			Experimental bacteremia induced by *E. coli, S. aureus, and C. albicans*	Commercial ELISA kit (Cusabio Technology, Houston, USA)	3 groups of 8 pigs each one (+control group: 8) Age: 10–15 weeks	Undetectable/very low levels of PCT in all groups, with no significant changes over time		Spyropoulos *et al.* (2019)
		Non-bacterial	*No research found*					
	Non-infectious[c]		*No research found*					
Horse	*Infectious*	*Bacterial*	Endotoxemia induced by *Lipopolysaccharide* inoculation	Commercial ELISA kit (MyBiosource, USA)	11 adult mares Age: 13.1±5.7 years	28.5 pg ml^{-1} (time 0, preinoculation)	Maximum concentration: 271 pg ml^{-1}	Bonelli *et al.* (2017)
			Enterocolitis with isolation of *Clostridium difficile*, *Salmonella spp*, or *Clostridium perfringens*	Commercial ELISA kit (Equine PCT, MyBioSource, San Diego, USA)	24 horses Age: 6 days to 60 months	15.34 pg ml^{-1}	252.21 pg ml^{-1}	El-Deeb *et al.* (2020)
			Sepsis (positive score)	In-house ELISA assay with human monoclonal antibodies	5 horses (+control group: 24) Age: 15±4 years	47 ng ml^{-1}	8450 ng ml^{-1}	Rieger *et al.* (2014)
		Non-bacterial	*No research found*					
	Non-infectious[c]		Colic with strangulating and non-strangulating intestinal lesions	Commercial ELISA kit (MyBioSource Inc, San Diego, CA, USA)	65 adult horses (+control group: 10) Age: 13.1±6.2 years	218.8 pg ml^{-1}	262.3 pg ml^{-1} in non-strangulating lesions, and 308.7 pg ml^{-1} in strangulating lesions	Kilcoyne *et al.* (2020)
Cattle	*Infectious*	*Bacterial*	Sepsis (pleuritis, pneumonia, diarrhea, peritonitis, omphalitis, polyarthritis)	Commercial ELISA kit (Bovine Procalcitonin ELISA kit, MyBiosource)	58 calves (+control group: 20) Age: 8.4±3.9 days	33.3 pg ml^{-1}	166.5 pg ml^{-1}	Bonelli *et al.* (2018)
			Septicemic colibacillosis	Commercial ELISA kit (Wuhan Huamei Biotech Co. Ltd., China)	15 neonatal calves (+control group: 15) Age: 2–10 days	46.2 pg ml^{-1}	175.6 pg ml^{-1}	Ercan *et al.* (2016)
		Non-bacterial	*No research found*					
	Non-infectious[c]		Left displacement of the abomasum (LDA)	Commercial ELISA kit (MyBioSource, USA)	30 dairy cows (+control group: 30)	6.4 ng l^{-1}	27 ng l^{-1}	Ismail *et al.* (2019)

[a]Values: results expressed as the arithmetic means of the values of the group unless otherwise stated.
[b]Control group: animals classified as healthy, unless specified that the selection procedure for the control group is different.
[c]Non-infectious disease: this category includes pathologies classified as non-infectious but not necessarily confirmed to be free of secondary infectious agents.

The membrane CD14 complex, thus the production of PSE, can be stimulated by PAMPs other than LPS during bacterial infections, and even from other ligands derived from virus and endogenous proinflammatory mediators, but it remains unknown to what extent (Poggi *et al.*, 2020). Such variabilities could explain the overlap in the concentrations reported in some studies between SIRS and sepsis (Behnes *et al.*, 2014), as well as the higher concentrations of PSE found in sepsis with a viral component or in endogenous damage such as kidney dysfunction (Chenevier-Gobeaux *et al.*, 2014; Zaninotto *et al.*, 2020). In addition, the origin of PSE could also explain why increases in its concentrations occur in infections with Gram-positive bacteria, but in lower magnitude than infections with Gram-negative bacteria that have LPS as a characteristic attribute (Koizumi *et al.*, 2020). In addition, PSE levels may be higher in some physiologic conditions like in preterm newborns and advanced age patients, in which concentrations reach 500 ng l^{-1}, the threshold at which the diagnosis of sepsis would be highly suspected in human beings. Therefore, more studies are needed to establish more accurate ranges based on age and associated diseases (Mussap *et al.*, 2012; Chenevier-Gobeaux *et al.*, 2014).

With respect to veterinary medicine, to the authors' knowledge, PSE has been only studied in horses. In one report, PSE concentrations did not change after the administration of LPS (Bonelli *et al.*, 2017). However, PSE was higher in horses classified as clinically endotoxemic patients from a veterinary teaching hospital (Fogle *et al.*, 2017) and there was an increase of PSE in foals with septicemia and in adult horses with recurrent airway obstruction (Wagner *et al.*, 2013). Therefore, more research is needed to explore the potential application of this promising biomarker in farm animals.

Other biomarkers that could have potential to detect sepsis

The following biomarkers could also have potential to detect sepsis, but have been studied less than PCT and PSE:

- *sTREM-1 (soluble Triggering Receptor Expressed on Myeloid Cells-1).* TREM-1 is a receptor of the immunoglobulin superfamily, whose expression is regulated on neutrophils, which mediates the inflammatory response triggered by bacteria and fungi (Bouchon *et al.*, 2001). TREM-1 is released in a soluble, quantifiable form (sTREM-1) from the membrane of activated cells. Higher concentrations of this biomarker during sepsis, and low expression of the receptor in patients with non-infectious inflammatory disorders, were observed among human patients (Gibot *et al.*, 2004). Regarding veterinary species, sTREM-1 has been measured in horses with inflammatory airway disease without significant changes (Bullone *et al.*, 2015), but there are no studies in animals with sepsis to the authors' knowledge.
- *Lipopolysaccharide-binding protein (LBP).* LBP is a protein that interacts with the LPS of Gram-negative bacteria and initiates a series of events that eventually lead to proinflammatory cytokine production. In several studies, LBP indicated infectious disease in human patients, but in other cases, this biomarker only distinguished infectious etiologies in patients with organ failure and its correlation with the severity of the disease was weak (Ouderkirk *et al.*, 2003; Sakr *et al.*, 2008). Regarding veterinary medicine, there is a study in which LBP increased significantly and faster than Hp in a group of calves experimentally infected with *Mannheimia haemolytica* (Schroedl *et al.*, 2001).
- *MMP-9.* Matrix-metalloproteinases (MMPs) are a family of endoproteinases involved in inflammation, healing of tissue injury, and remodeling. MMP-9 concentrations rise in people with severe sepsis, and its analysis could help determining the outcome of the illness (Hoffmann *et al.*, 2006, 2009). In veterinary medicine, MMP-9 has been detected at higher activity after the administration of LPS in horses and pigs (Castellheim *et al.*, 2008; Fugler *et al.*, 2013). In addition, higher concentrations of MMP-9 have been found in bronchoalveolar lavage fluid in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV) and in calves with concurrent infections of *Pasteurella multocida* and *Mycoplasma bovirhinis* (Girard *et al.*, 2001; Simonen-Jokinen *et al.*, 2005). In cattle, higher activity in milk has also been reported after endotoxin-induced mastitis and in synovial fluid after an experimentally induced septic arthritis (Raulo *et al.*, 2002; Francoz *et al.*, 2008). Furthermore, MMP-9 can be covalently linked to Hp and released as MMP9-Hp complexes by neutrophils when their degranulation occurs. These complexes were firstly detected in the serum of cattle with acute polymicrobial sepsis (Bannikov *et al.*, 2007, 2011) and then later found at high concentrations after the infusion of LPS (Hinds *et al.*, 2014).

Biomarkers that can provide additional information in sepsis

There are other biomarkers that could be useful in diagnosed cases of sepsis, for establishing the patient's prognosis by assessing endothelial damage, organ dysfunction, and alterations in the coagulation system.

- *Markers of endothelial damage.* The endothelial system plays an essential role in homeostasis. Endothelial damage and dysfunction can lead to complications that often accompany sepsis, such as organ dysfunction and septic shock. Examples of endothelial damage biomarkers are neopterin, angiopoietins, endocan, vascular cell adhesion molecule-1, vascular endothelial growth factor, platelet-derived growth factor, or adrenomedullin (Christ-crain *et al.*, 2005; Pierrakos and Vincent, 2010; Paulus *et al.*, 2011; Giannakopoulos *et al.*, 2017). In veterinary medicine, neopterin was significantly higher in calves with septicemic colibacillosis (Ercan *et al.*, 2016), and adrenomedullin was found higher in critically ill neonatal foals regardless of the causal agent (Toth *et al.*, 2014).
- *Markers of organic dysfunction.* Some examples of these biomarkers are lactate, endothelin-1 (ET-1), and natriuretic peptides such as atrial natriuretic peptide and mid-regional pro-atrial natriuretic peptide. These markers could be valuable as early predictors of high-risk patients and survival likelihood (Brauner *et al.*, 2000; Rivers *et al.*, 2007; Mikkelsen *et al.*, 2009). In animals, an increase in lactate was reported after the experimental induction of sepsis in pigs, and this marker was correlated with a higher mortality rates in horses with SIRS (Corley *et al.*, 2010; Tóth *et al.*, 2017). Regarding ET-1, higher plasma concentrations and mRNA levels in tissues were observed after the experimental induction of sepsis in pigs (Zannoni *et al.*, 2010).
- *Markers of the coagulation system.* In sepsis, it is frequent to observe changes in thrombocyte counts, antithrombin, protein C and S, activated partial thromboplastin time, D-dimer, or fibrin. These changes are not specific and can also occur in

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other pathologies such as cancer, trauma, and vascular or immune disorders, but their study during sepsis is helpful for prognosis and evaluation of the treatment (Pettilä *et al.*, 2002). In horses and cattle, the coagulation markers that seem more useful are fibrinogen and D-dimer (Giguère *et al.*, 2003; Irmak *et al.*, 2006; Armengou *et al.*, 2008; Hultén *et al.*, 2010), but further research is needed.

Conclusions

This review has described the general concepts of sepsis and the current knowledge about three groups of potential biomarkers of sepsis in pigs, horses and cattle, namely: (1) APPs and cytokines, which have been traditionally used; (2) PCT, PSE, and other proteins that are more specific sepsis markers; and (3) other markers that can provide complementary information about this condition.

Overall, each biomarker of the three groups could have an use in sepsis and provide complementary information. APPs and cytokines, although non-specific for sepsis, are indicative of the inflammation associated with this condition and can monitor the evolution of the disease and establish the prognosis of the patient. PCT, PSE, and other molecules such as sTREM-1, LBP, and MMP-9, have huge potential because they can improve the diagnosis of bacterial sepsis. Finally, the biomarkers of the third group provide additional information for prognosis and monitoring through the assessment of endothelial damage, organic dysfunction, and alterations in the coagulation system.

Further studies should better define the ability and applications of these biomarkers, especially those with potential to differentiate sepsis from other inflammatory conditions, and establish accurate cut-off values in veterinary medicine. These are required steps to improve the diagnosis and the clinical management of sepsis in veterinary species, which is essential to increase host survival rates and achieve more rational use of antibiotics.

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Objective 2

Identification of new potential biomarkers using proteomics techniques in:

- **An experimental model of septic and non-septic inflammation through administering LPS of *E. coli* and turpentine oil to pigs.**
- **Samples from commercial farms with meningitis due to *S. suis.***
- **Samples from commercial farms with diarrhoea caused by *E. coli.***

Article 2 (published):

A proteomic approach to elucidate the changes in saliva and serum proteins of pigs with septic and non-septic inflammation

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Article

A Proteomic Approach to Elucidate the Changes in Saliva and Serum Proteins of Pigs with Septic and Non-Septic Inflammation

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Abstract: Sepsis is a systemic inflammatory response triggered by an infectious agent and is recognized by the World Health Organization as a global concern, since it is one of the major causes of severe illness in humans and animals. The study of the changes that can occur in saliva and serum in sepsis can contribute to a better understanding of the pathophysiological mechanisms involved in the process and also to discover potential biomarkers that can help in its diagnosis and monitoring. The objective of this study was to characterize the changes that occur in the salivary and serum proteome of pigs with experimentally-induced sepsis. The study included five pigs with sepsis induced by LPS administration and five pigs with non-septic inflammation induced by turpentine for comparative purposes. In saliva, there were eighteen salivary proteins differentially expressed in the sepsis condition and nine in non-septic inflammation. Among these, significant increments in aldolase A and serpin B12 only occurred in the sepsis model. Changes in aldolase A were validated in a larger population of pigs with sepsis due to *Streptococcus suis* infection. In serum, there were 30 proteins differentially expressed in sepsis group and 26 proteins in the non-septic group, and most of the proteins that changed in both groups were related to non-specific inflammation. In the saliva of the septic animals there were some specific pathways activated, such as the organonitrogen compound metabolic process and lipid transport, whereas, in the serum, one of the main activated pathways was the regulation of protein secretion. Overall, saliva and serum showed different proteome variations in response to septic inflammation and could provide complementary information about the pathophysiological mechanisms occurring in this condition. Additionally, salivary aldolase A could be a potential biomarker of sepsis in pigs that should be confirmed in a larger population.

Keywords: sepsis; proteomics; pigs; saliva; serum

1. Introduction

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) triggered by an infectious agent [1]. It has been recognized as a global priority by the World Health Organization and the World Health Assembly [2], as it is one of the major causes of severe illness in humans and animals [3,4]. The most challenging aspect of sepsis is its diagnosis

and distinction from SIRS, which does not always involve infection but also non-infectious conditions, like trauma [5]. The misdiagnosis of a non-septic inflammation as sepsis results in a non-rational use of antibiotics, which enhances the development of multidrug-resistant bacteria leading to a "One Health" problem [6,7]. The study of the protein variations that can occur in saliva and serum in sepsis could contribute to a better knowledge of the pathophysiology of sepsis and also aid in the discovery of new potential biomarkers for this condition. This knowledge is of importance in the context of the reduction of antibiotic resistances [8], because could lead to a more accurate identification of this condition and, therefore, the adequate and reasonable use of antibiotics, which contributes to the reduction of antibiotic resistance [9,10]. This is particularly important in the pig, as it is the domestic species in which antibiotics are most used [11].

The administration of lipopolysaccharide (LPS) from Gram-negative pathogens is a recognized experimental model of sepsis induction in the pig [12]. In addition, non-septic inflammation can be induced in this species with the experimental injection of turpentine oil [13]. The usefulness of animal models in the study of sepsis has been controversial, based on the reasoning that these models do not mimic human clinical sepsis in which a hyperdynamic state occurs, in contrast to the decreased cardiac output and increased systemic vascular resistance that happens after LPS administration in animal models [14]. Another cause for concern is the balance between the benefits obtained from the study of the pathophysiology of sepsis and the well-being of the animals implicated in these experimental models [15]. However, LPS administration is considered an adequate model in pigs to study the pathophysiology of inflammation, immune system [13,16,17], and changes in biomarkers of oxidative status [18] associated with sepsis.

Analyses of saliva samples are gaining growing importance in pigs as a sample to evaluate health status since they can be collected by non-invasive procedures and with less stress compared to venipuncture [19]. In the previous studies about the inflammatory response triggered by the repeated administration of LPS [20] or the local application of turpentine [21], acute-phase proteins (e.g., C-reactive protein and haptoglobin) and stress indicators (e.g., cortisol) were reliably detected in saliva samples.

The application of proteomics allows the study of a complete protein profile of a sample, evidencing the specific alterations associated with specific metabolic pathways [22]. Gel-based proteomics has been already used to investigate the serum proteome of swine after LPS administration [23]. Nevertheless, the gel-free mass spectrometry-based proteomics has the advantage of being more sensitive and allows the high-throughput profiling of proteins providing higher quantification accuracy than the gel-based techniques [24]. The use of isobaric tagging with tandem mass tags (TMT) allows the simultaneous relative quantification of differentially labeled peptides, increasing the sensitivity of the gel-free proteomics analysis [25].

To the best of authors' knowledge, there are no studies about proteomic in porcine saliva in pigs with sepsis. The objective of this report was to study the sepsis-related changes in the proteome of saliva and serum in the pig, with the ultimate goal of broadening the understanding of the pathophysiological mechanisms involved in sepsis and detecting possible potential biomarkers. For this purpose, we investigated the dynamic changes of proteins in saliva and serum in pigs in a model of septic inflammation induced by the administration of LPS. In addition, we used a model of non-septic inflammation through the administration of turpentine oil for comparative purposes.

2. Results

2.1. Proteomic Changes in LPS-Challenged Pigs

A total of 18 proteins showed a different relative abundance in the saliva of pigs after LPS administration (Table 1). Among these, the proteins with the highest change were fructose-biphosphate aldolase (ALDOA), serpin domain-containing protein

(SERPINB12), annexin (ANXA2), moesin (MSN), and the immunoglobulins M (IgM) and G (IgG), all being upregulated. The higher magnitude of increase of these proteins was observed at 6 h post-LPS administration.

Table 1. Differentially expressed salivary proteins in pigs with experimentally-induced sepsis.

Gene (or Accession Number)	Protein Name	Mean Abundances			Fold Changes	
		Basal	6 h	24 h	6 h/Basal	24 h/Basal
ALDOA	Fructose-biphosphate aldolase	0.50	1.13	0.88	1.18 **	0.83
SERPINB12	SERPIN domain-containing protein	0.42	0.92	0.74	1.12 *	0.80
ANXA2	Annexin	0.50	1.02	0.65	1.04 *	0.39
SFN	14-3-3 sigma protein	0.61	1.23	0.94	1.02 **	0.63
MSN	Moesin	0.67	1.34	1.12	0.99 *	0.74
SERPINB1	Leukocyte elastase inhibitor	0.74	1.35	0.97	0.88 *	0.39
IGHA	IgM	0.64	1.16	0.97	0.86 **	0.59
ECH1	Galectin	0.61	1.07	0.93	0.81 *	0.60
FABP5	FABP domain-containing protein	0.66	1.06	1.16	0.70	0.82 *
A2M	Alpha-2-macroglobulin isoform a	0.80	1.28	1.01	0.68 **	0.33
IGHG	IgG heavy chain	0.82	1.28	0.80	0.63 *	-0.04
LMNA	Lamin isoform A	0.74	1.11	1.01	0.59 *	0.45
P4HB	Protein disulfide-isomerase	0.79	1.19	1.06	0.59 *	0.43
TKT	Transketolase	0.75	1.13	1.03	0.59 *	0.45
YWHAZ	14-3-3 protein zeta/delta	0.64	0.86	1.13	0.43	0.83 *
CSTB	Cystatin-B	0.80	1.06	1.35	0.40	0.75 *
LCN2	Neutrophil gelatinase-associated lipocalin	0.72	0.81	1.31	0.18	0.86 *
P51524 (accession)	Prophenin and tritrpticin precursor (Fragment)	0.73	0.54	1.71	−0.43	1.23 *

* p-value < 0.05; ** p-value < 0.01.

GO enrichment analysis indicated that the differentially expressed salivary proteins in LPS-challenged pigs were significantly associated with 11 different GO terms (Supplementary Table S1), namely, the organonitrogen compound metabolic process (GO:1901564), tissue development (GO:0009888), the regulation of the developmental process (GO:0050793), and lipid transport (GO:0006869) (Figure 1).

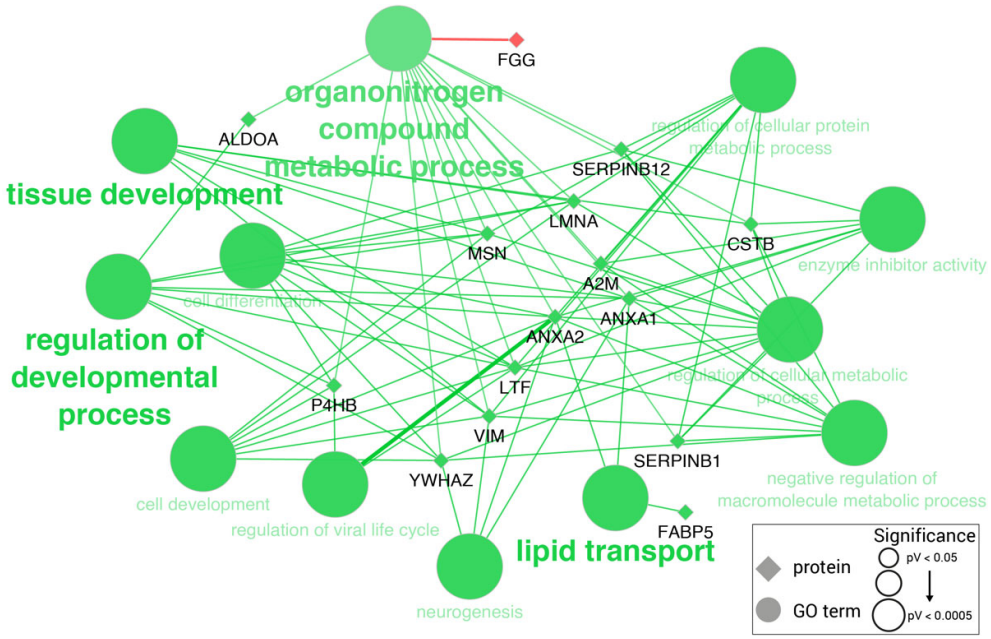

Figure 1. Significantly enriched GO terms among differentially expressed proteins in the saliva of pigs with sepsis. Edges link proteins (diamonds) to their associated GO terms (circles). Proteins are colored in green if overexpressed or in red if down-expressed. GO terms are colored accordingly to the proportion of over-/down-expressed proteins. GO term shape and font size is proportional to GO term significance, but all included ones showed a p-value < 0.05.

The serum protein profile of the LPS-challenged pigs showed significant differences in the abundances of 30 proteins after LPS administration (Table 2). Among those with the higher relative abundance, the most protruding changes at 24 h were present in two SERPIN domain-containing proteins (LOC106504547 and LOC396684), followed by haptoglobin (HP), pentraxin/C-reactive protein (CRP) and apolipoprotein (APOE). At 24 h, the proteins with lowest relative abundance were thrombospondin 1 (THBS1), vitronectin (VTN), plasma retinol-binding protein (RBP4), carboxypeptidase B2 (CPB2), and the apolipoprotein A-1 (APOA1). Although these proteins showed reduced abundance levels at 6 h post-LPS administration, they reached their lowest levels at 24 h.

Table 2. Differentially expressed serum proteins in pigs with experimentally-induced sepsis.

Gene (or Accession Number)	Protein Name	Mean Abundances			Fold Changes	
		Basal	6 h	24 h	6 h/Basal	24 h/Basal
LOC106504547	SERPIN domain-containing protein	0.78	1.02	1.66	0.43	1.13 ***
LOC396684	SERPIN domain-containing protein	0.90	1.07	1.51	0.25	0.74 **
HP	Haptoglobin	0.77	0.98	1.21	0.34	0.63 **
CRP	Pentaxin/C-reactive protein	0.56	1.09	0.86	0.96 **	0.62
APOE	Apolipoprotein E	0.97	1.14	1.04	0.23 *	0.10
LBP	Lipopolysaccharide-binding protein	0.84	0.95	1.08	0.17	0.36 *
LUM	Lumican	0.94	0.99	1.18	0.07	0.32 *

A0A480XY00	Complement C1s subcomponent isoform 1 preproprotein	0.98	0.98	1.10	−0.01	0.16 **
FGB	Fibrinogen beta chain	0.94	0.78	1.10	−0.26 **	0.22
FGG	Fibrinogen C-terminal domain-containing	0.99	0.81	1.06	−0.29 *	0.10
FGA	Fibrinogen alpha chain	0.96	0.78	1.02	−0.29 *	0.08
FN1	Fibronectin	1.03	0.91	0.95	−018 *	−0.12
ALB	Albumin	1.01	1.00	0.93	−0.02	−0.12 *
SERPINC1	Antithrombin-III	0.99	0.94	0.90	−0.06	−0.13 *
SERPINA7	Thyroxine-binding globulin	1.02	0.99	0.92	−0.10	−0.14 *
ITIH1	Inter-alpha-trypsin inhibitor heavy chain H1 isoform a preproprotein	1.06	1.00	0.95	−0.08	−0.14 *
C8B	Complement component 8 subunit beta	1.01	1.04	0.90	0.04	−016 *
PROC	Vitamin K-dependent protein C	1.16	1.08	1.02	−010	−0.19 *
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	1.07	1.02	0.92	−0.06	−0.22 *
A0A4X1TBX0	C1q domain-containing protein	1.01	0.95	0.86	−0.08	−0.22 *
C8G	Complement component C8G	1.03	1.04	0.88	0.01	−0.23 *
AFM	Afamin	1.09	1.03	0.92	−0.07	−0.24 *
SERPINA6	SERPIN domain-contaning protein	1.03	1.03	0.85	−0.01	−0.27 *
PLG	Plasminogen	1.02	1.00	0.84	−0.02	−0.27 **
GSN	Actin-depolymerizing factor	1.06	1.05	0.87	−0.01	−0.27 *
FETUB	Fetuin-B isoform 1	1.11	0.99	0.01	−0.17	−0.28 **
HRG	Histidine-rich glycoprotein	1.00	0.97	0.80	−0.03	−0.31 **
APOA1	Apolipoprotein A-1	1.13	1.02	0.90	−0.15	−0.32 *
CPB2	Carboxypeptidase B2 isoform 1 preproprotein	1.06	1.06	0.84	-0.01	−0.32 **
VTN	Vitronectin	1.09	0.92	0.82	−0.24	−0.40 **

* p-value < 0.05; ** p-value < 0.01..

GO analysis enriched 34 GO terms among the altered serum proteins (full list in Supplementary Table S2). Downregulated proteins were more associated with cysteine-type endopeptidase activity (GO:0004197), vitamin transport (GO:0051180) or negative regulation of cell adhesion (GO:0007162), while upregulated proteins were more associated with establishment of localization to extracellular region (GO:0035592) and the regulation of protein secretion (GO:0050708). Moreover, in general, both types of proteins were functionally related to the upregulation of different biological processes, in particular, the negative regulation of biological processes (GO:0048519), the negative regulation of blood coagulation (GO:0030195), as well as the regulation of the response to external stimulus (GO:0032101) and the regulation of the apoptotic process (GO:0042981) among others (Figure 2).

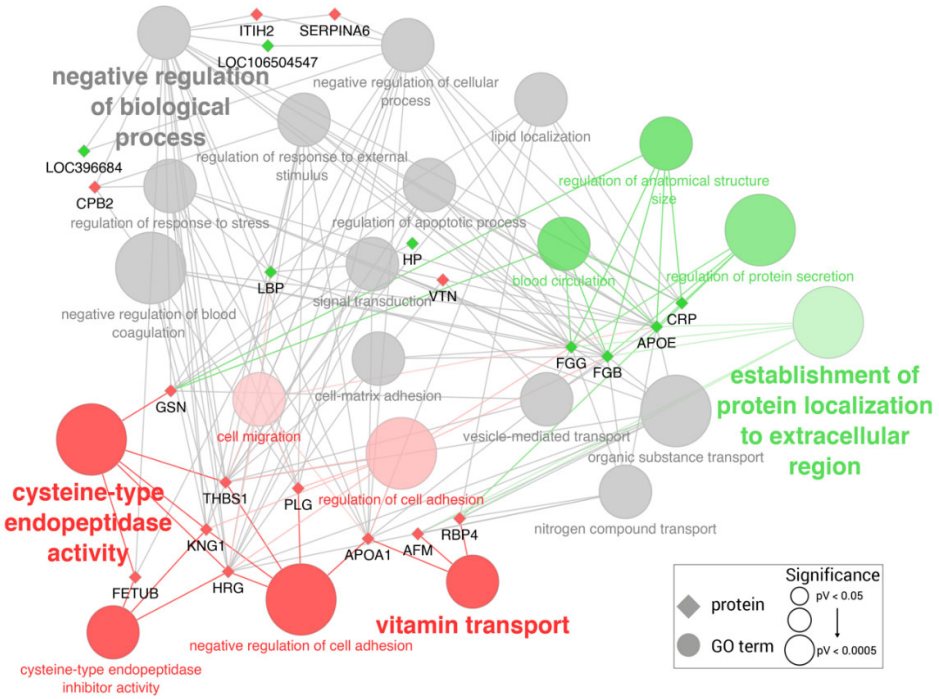

Figure 2. Significantly enriched GO terms among differentially expressed proteins in the serum of pigs with sepsis. Edges link proteins (diamonds) to their associated GO terms (circles). Proteins are colored in green if overexpressed or in red if down-expressed. GO terms are colored accordingly to the proportion of over-/down-expressed proteins. GO term shape and font size is proportional to GO term significance, but all included ones showed a p value < 0.05.

2.2. Proteomic Changes in Turpentine-Challenged Pigs

A total of nine salivary proteins showed significantly increased abundance in pigs after turpentine administration (Table 3), while no protein showed significant decreases. The most significant increases were observed in albumin (ALB), a H4 histone (UniProtKB: P51524), and a cystatin domain-containing protein (HRG), among others. The higher upregulation levels were detected at 6 h after turpentine administration.

Table 3. Differentially expressed salivary proteins in pigs with experimentally-induced non-septic inflammation.

		Mean Abundances			Fold Changes	
Gene	**Protein Name**	**Basal**	**6 h**	**24 h**	**6 h/Basal**	**24 h/Basal**
P62802	Histone H4	0.49	1.13	0.73	1.18 *	0.55
ALB	Albumin	0.54	1.12	0.76	1.05 **	0.50
HRG	Cystatin domain-containing protein	0.67	1.25	0.90	0.88 *	0.42
A2M	Alpha-2-macroglobulin isoform a	0.61	1.03	0.73	0.75 *	0.24
TF	Beta-1 metal-binding globulin	0.72	1.14	0.93	0.65 **	0.37
IGHG	IgG heavy chain	0.67	1.05	0.90	0.64 *	0.42
P51524 (accession)	Prophenin and tritrpticin precursor (Fragment)	0.54	0.82	1.46	0.58	1.41 *
LOC106504547	SERPIN domain-containing protein	0.69	0.90	1.15	0.37	0.73 **
LCN2	Neutrophil gelatinase-associated lipocalin	0.70	0.89	1.17	0.34	0.72 *

* p-value < 0.05; ** p-value < 0.01.

GO enrichment analysis showed significant over-representation of the GO terms: antimicrobial humoral response (GO:0019730), focal adhesion assembly (GO:0048041), and serine-type endopeptidase activity (GO:0004252) (Figure 3) (full list in Supplementary Table S3).

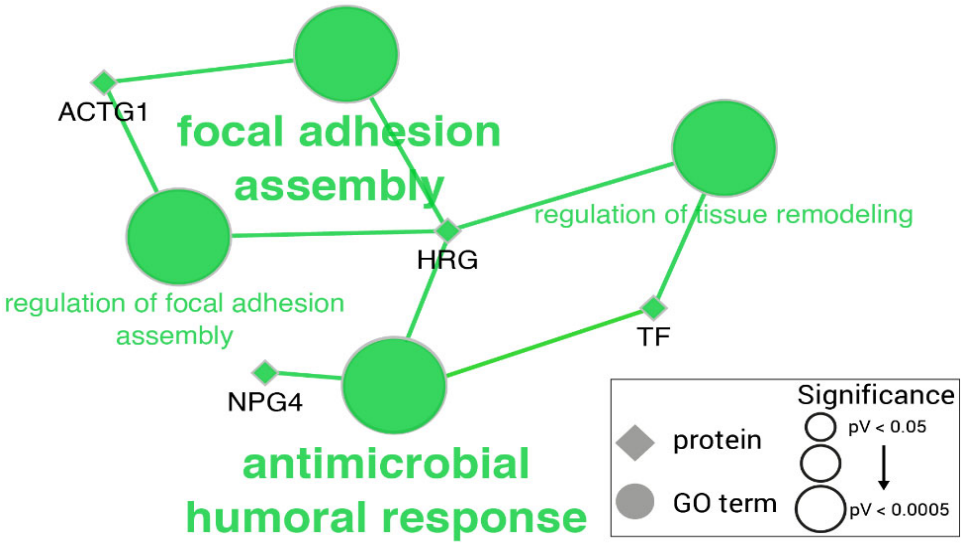

Figure 3. Significantly enriched GO terms among differentially expressed proteins in the saliva of pigs with non-septic inflammation. Edges link proteins (diamonds) to their associated GO terms (circles). Proteins colored in green are overexpressed. GO terms are colored accordingly to the proportion of over-/down-expressed proteins. GO term shape and font size is proportional to GO term significance, but all included ones showed a p value < 0.05.

In serum, a change in the relative abundance after turpentine administration was noted for 26 proteins (Table 4). The most upregulated proteins were CRP, two SERPIN domain-containing proteins (LOC106504547 and LOC100156325), HP, fibrinogen alpha chain (FGA), and the lipopolysaccharide-binding protein (LBP), showing their higher expressions levels 24 h post-turpentine administration. On the other hand, the most downregulated proteins overall after 24 h were APOA1, MACPF domain-containing protein (C8A), SERPINA6, plasma retinol (RBP4), and vitronectin (VTN).

Table 4. Differentially expressed serum proteins in pigs with experimentally-induced non-septic inflammation.

		Mean Abundances			Fold Changes	
Gene (or Accession Number)	**Protein Name**	**Basal**	**6 h**	**24 h**	**6 h/Basal**	**24 h/Basal**
CRP	Pentaxin or C-reactive protein	0.46	0.66	1.30	0.50	1.47 *
LOC106504547	SERPIN domain-containing protein	0.688	0.73	1.74	0.08	1.32 **
LOC100156325	SERPIN domain-containing protein	0.69	0.83	1.13	0.27	0.71 *
HP	Haptoglobin	0.80	0.87	1.29	0.10	0.67 **
FGA	Fibrinogen alpha-chain	0.85	0.91	1.32	0.09	0.62 **
LBP	Lipopolysaccharide-binding protein	0.90	0.98	1.34	0.11	0.57 **
FGB	Fibrinogen beta chain	0.86	0.91	1.19	0.09	0.47 **

FGG	Fibrinogen C-terminal domain-containing protein	0.91	0.93	1.21	0.02	0.42 **
A0A4X1U9T5 (accession)	Ig-like domain-containing protein	0.97	0.95	1.11	−0.02	0.19 *
APOA1	Apolipoprotein A-1	1.25	1.13	0.72	−0.13	−0.8 **
C8A	MACPF domain-containing protein	1.27	1.11	0.90	−0.19	−0.48 *
RBP4	Plasma retinol-binding protein	1.10	1.07	0.79	−0.03	−0.47 *
SERPINA6	SERPIN domain-containing protein	1.12	1.01	0.82	−0.14	−0.45**
VTN	Vitronectin	1.19	1.16	0.92	−0.03	−0.36 **
APON	Ovarian and testicular apolipoprotein N	1.08	0.99	0.85	−0.13	−0.34 **
ITIH1	Inter-alpha-trypsin inhibitor heavy chain H1 isoform a preproprotein	1.01	1.03	0.83	0.02	−0.29 **
HRG	Histidine-rich glycoprotein	1.09	1.04	0.89	−0.06	−0.28 *
GSN	Actin-depolymerizing factor	1.03	0.99	0.88	−0.05	−0.23 *
FETUB	Fetuin-B isoform 1	1.11	1.09	0.95	−0.02	−0.23 *
TF	Serotransferrin	1.06	1.07	0.91	0.01	−0.22 *
A0SEH3 (accession)	Complement component C8G	1.00	0.98	0.88	−0.03	−0.18 *
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	1.05	1.03	0.92	−0.02	−0.18 *
AMBP	Alpha-1-microglobulin	1.01	0.98	0.89	−0.04	−0.18 *
PROC	Vitamin K-dependent protein	1.10	1.07	0.99	−0.04	−0.15 *
A1BG	Alpha-1B-glycoprotein	1.06	1.04	0.97	−0.01	−0.12 *
A1BG	Alpha-1B-glycoprotein	1.06	1.04	0.97	−0.01	−0.12 *

* p-value < 0.05; ** p-value < 0.01.

GO enrichment analysis indicated that downregulated proteins have functions related to cysteine-type endopeptidase activity (GO:0004197) and actin cytoskeleton organization (GO:0030036), while upregulated proteins participate in vasoconstriction (GO:0042310), protein polymerization (GO:0051258), and the positive regulation of peptide hormone secretion (GO:0090277). Both are equally associated with hormone transport (GO:0009914) and response to chemicals (GO:0042221) (Figure 4) (full list in Supplementary Table S4).

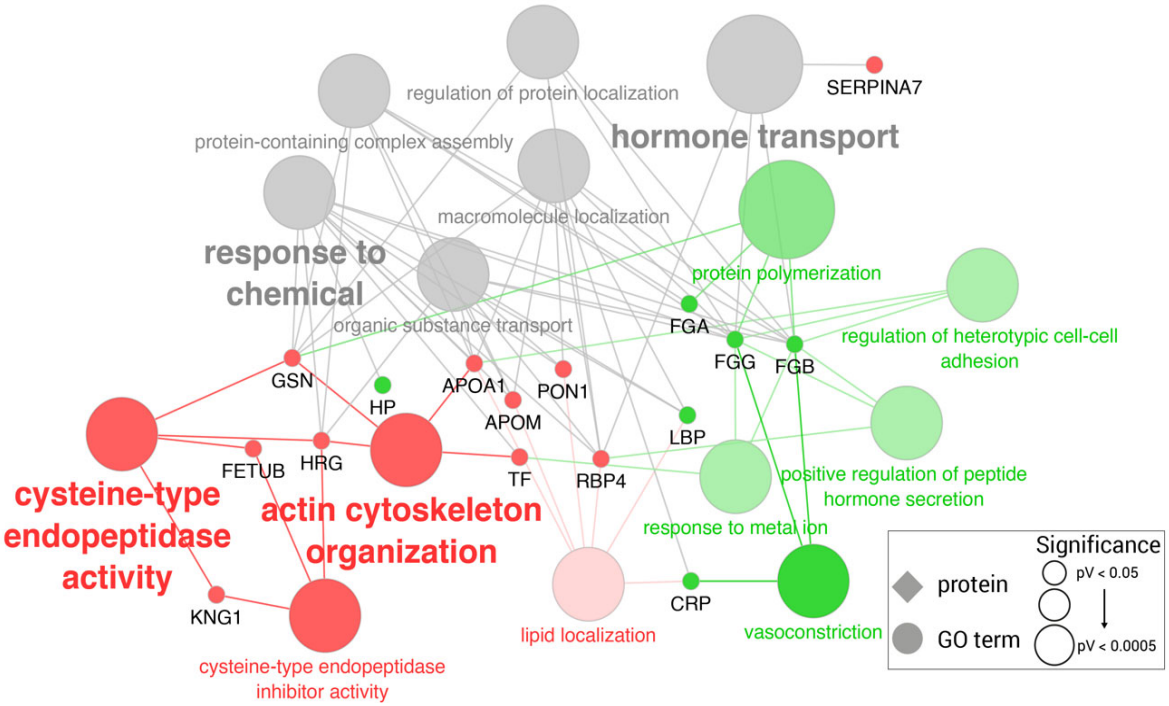

Figure 4. Significantly enriched GO terms among differentially expressed proteins in the serum of pigs with non-septic inflammation. Edges link proteins (diamonds) to their associated GO terms (circles). Proteins are colored in green if overexpressed or in red if down-expressed. GO terms are colored accordingly to the proportion of over-/down-expressed proteins. GO term shape and font size is proportional to GO term significance, but all included ones showed a p value < 0.05.

2.3. *Measurement of Aldolase Activity in Porcine Saliva*

The analytical validation of the ALDOA automated assay showed an intra- and inter-assay imprecision less than 10% and a high linearity (R > 0.99) after the serial dilutions of a saliva sample with high ALDOA activity. The lower limit of quantification (LLOQ) and the limit of detection (LoD) were set at 1.3 and 0.1 U/L, respectively.

The activity of salivary ALDOA was significantly higher in pigs after LPS-induced sepsis at 6 h post injection (median 7.1 U/L and range 5.2–7.3 U/L) compared with basal values (median 2.1 U/L and 1.4–3.2 U/L) (P = 0.020), while at 24 h the ALDOA activity (median 6.2 U/L and range 3.2–8.5 U/L) did not show significant differences compared with basal values (Figure 5A). In the case of the turpentine-induced group, ALDOA activity at 6 h (median 2.2 U/L and range 1.4–3.8 U/L) and at 24 h (median 2.5 U/L and range 0.4–4.5 U/L) showed no significant differences in comparison with the basal values (median 1.3 U/L and range 1.1–3.6 U/L) (Figure 5B). No significant differences in ALDOA activities were observed between males and females in the two-way ANOVA analysis.

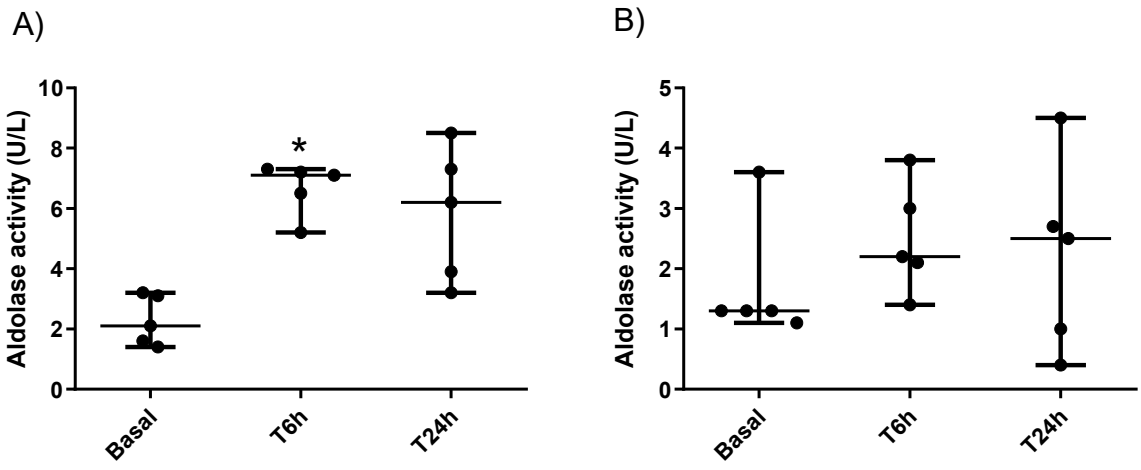

Figure 5. Salivary aldolase activity levels (U/L) during experimentally-induced sepsis in a model of LPS-challenged pigs (**A**) and non-septic inflammation in a model of turpentine-challenged pigs (**B**). Basal: 24 h before LPS- or turpentine-injection; T6 h: 6 h after LPS- or turpentine-injection; T24 h: 24 h after LPS- or turpentine-injection. Lines indicate the minimum, median, and maximum values. Asterisks indicate statistically significant differences (*$p < 0.05$) with basal time. Circles represent the sample values .

In addition, pigs with meningitis showed significantly higher activity levels of salivary ALDOA (median 10.20 U/L and range 8.1–15.1 U/L) compared with healthy controls (median 2.80 and range 0.2–9.8 U/L) ($p = 0.001$) (Figure 6).

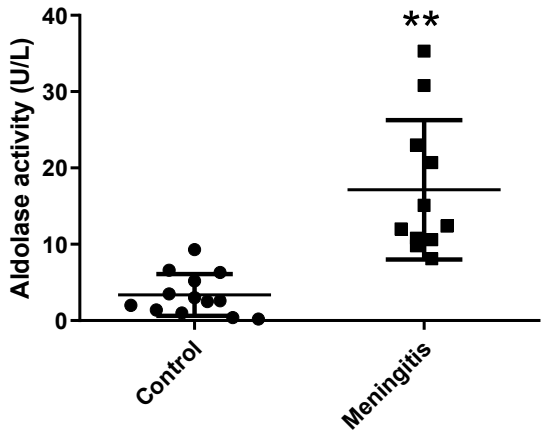

Figure 6. Salivary aldolase activity (U/L) in pigs with meningitis compared with healthy controls. Lines indicate the minimum, median, and maximum values. Asterisks indicate statistically significant differences (**$p = 0.001$). Circles and squares represent the sample values of control and meningitis groups, respectively.

3. Discussion

In this study, we reported the changes in salivary and serum protein profiles of pigs with septic inflammation experimentally-induced by LPS administration and their pathophysiological implications. We observed a higher number of proteins changed in abundance in the saliva of the sepsis-induced group compared with the non-septic inflammation group (18 vs. 9). In addition, some of the proteins that changed in the pigs with sepsis did not have significant changes in non-septic conditions. For example, in

sepsis, ALDOA, and serpin B12, which were the proteins that showed higher increases, did not display significant changes in the turpentine model.

ALDOA is a glycolytic enzyme catalyzing the reversible cleavage of fructose-1, 6-bisphosphate (Fru-1, 6-P2) into glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). The family of ALDOA, in addition to be integrated by glycolytic enzymes, has a close relationship with muscle damage, the development of the brain, and ATP production. ALDOA increases its expression in the muscles of rats treated with LPS [26,27] and is present in the membrane surface of different pathogens where it acts as an adhesin [28,29]. Therefore, ALDOA production is directly related to the presence of sepsis by acting as a promoter of bacterial adhesion to the host cell receptors to facilitate invasion [30], thus being a potent stimulator of immune response in humans [31].

When ALDOA was measured by an automated spectrophotometric assay, it showed significant increases in pigs after the LPS administration at 6 h post-injection, as occurred in the proteomic study. In addition, our validation study found that ALDOA was higher in pigs naturally affected by meningoencephalitis caused by S. suis compared with healthy pigs. This result could reinforce the possibility of considering the salivary ALDOA as a possible biomarker of sepsis in field conditions. Gender had no influence on the ALDOA activity in the validation study. However, these results should be taken with caution and should be verified in a large population of pigs and also in pigs with sepsis produced by different infectious agents.

Serpin B12 is an inhibitor of trypsin-like serine proteinases as trypsin and plasmin [32]. The presence of this protein in epithelia and tissues suggests a role in host defense, either by the inhibition of exogenous viral and bacterial proteases or by a cytoprotective role of vital cells from endogenous proteases needed to combat infection [33]. Therefore, it could be related to the defense and compensatory effects of the organism against bacterial infection.

In this study, we also identified a set of proteins in which abundance was significantly changed in the saliva of pigs with non-septic inflammation induced by turpentine. Two specific proteins were upregulated in non-septic inflammation but showed no significant variations in the saliva of pigs with sepsis: ALB and histone H4. ALB was detected in human saliva as a part of plasma derivates or the production of salivary glands [34]. We hypothesized that the presence of albumin in the saliva of pigs could be more related to the local production of this protein than to the ultrafiltration of serum since the pigs subjected to non-septic inflammation did not show elevated levels of albumin in the serum. Further studies should be performed to clarify the reason for the increase in salivary albumin in non-septic inflammation in pigs and evaluate its use as a possible marker of this condition.

We also detected increased abundances of histone H4 in non-septic inflammation. Histones are basic proteins located in the nucleus. It is believed that histones play proinflammatory functions upon their release from the nucleus into the extracellular environment. Previous studies [35] reported their elevated levels in sepsis causing cellular injury, which creates an additional research area because our results include the elevations only in the turpentine model.

The differences found in GO analysis in the saliva of pigs with sepsis were associated with the lipid transport, tissue development, and organonitrogen compound metabolic process. Previous reports have indicated the involvement of lipids in the pathogenesis of sepsis, with lipid moieties playing a role in pathogen toxin clearance and in modulating inflammatory responses [36]. On the other hand, changes in humoral immune response and serine-type endopeptidase activity appeared in non-septic inflammation.

In the serum of both LPS and turpentine groups, the proteomic analysis showed that pigs experienced an increase in HAPT and CRP at 6 h post-stimulus. However, these proteins were not detected in saliva by proteomics, and this could be due to the low concentrations of these proteins in this fluid. Our results in serum are in line with a previous report, in which the serum proteome of swine after LPS administration was

analyzed [23]. The authors indicated the upregulation of HAPT and CRP after 6 h post LPS injection. These results were further confirmed by ELISA assays and are in agreement with other studies which showed that serum concentrations of CRP and HAPT can rapidly increase during the first 4–5 h after exposure to a single stimulus [37,38]. One protein that showed differences in serum abundance between LPS- and turpentine-challenged pigs was apolipoprotein E (APOE), which showed increases in the group of LPS. The increase of this protein has been related to the risk of sepsis in human beings [39].

Serum and saliva showed different responses in our experimental conditions, with differences in the number of proteins showing significant changes as well as in the pattern of proteins with variations. These discrepancies in the number and types of proteins that change between these fluids have been observed previously in other species and diseases [40–43]. Therefore, saliva and serum could present complementary information when analyzed.

This study has some limitations that must be considered. First, we employed a small number of animals, and therefore this study should be considered as a pilot and additional studies with a larger population of animals and with different septic diseases should be carried out to confirm our findings. Additionally, gender had no influence on the ALDOA activity in the validation study, but these results should be taken with caution and should be verified in a large population of pigs and also in pigs with sepsis produced by different infectious agents. In addition, we used the basal times of each group as a reference for ideal health status but, ideally, a control group of pigs with no treatment might be included. In order to avoid the stress of a blood extraction to the pigs, only saliva was used in the validation study and, ideally, the proteomic results should also be validated in the serum of pigs with meningitis. Furthermore, the changes in the proteome of pigs with sepsis and non-septic inflammation might be accompanied by changes in the transcriptome that have not been assessed in this study and would be of interest for further research.

4. Materials and Methods

4.1. Animals

4.1.1. Proteomic Study

Samples from growing male pigs (*Sus scrofa domesticus*) (Large White) in the mid fattening period from the Experimental Farm of the University of Murcia (Murcia, Spain) were used in this study (full information of animals can be found in Supplementary Table S5). The experimental procedure used to obtain these samples was described in a previous report [44]. The samples were from the following two groups:

- Lipopolisacharide (LPS) group ($n = 5$). Pigs were individually administered LPS from Escherichia coli (Sigma-Aldrich, St. Louis, MO, USA) reconstituted in sterile saline solution in a single dose of 30 ug/kg by intramuscular route as previously reported [45].
- Turpentine group ($n = 5$). Each pig was administered a total of 8 mL subcutaneous injections of turpentine oil (oil of turpentine purified, Sigma–Aldrich, St. Louis, MO, USA), 4 mL in each front flank, as previously described [46].

In all animals, LPS and the turpentine oil were administered between 8 am and 9 am. The saliva and blood samples from three collection times were analyzed in the proteomic study: 24 h before the experiment (basal) and 6 h (T6) and 24 h (T24) post-administration.

4.1.2. Validation Study

For the validation study, we analyzed the changes in the saliva level of the proteins selected after the proteomic study in two different situations:

1. Septic and non-septic experimentally-induced inflammation: An aliquot of each saliva sample of the LPS and turpentine groups used in the proteomic study was analyzed.
2. Sepsis in field conditions: Two groups of Large White weaning pigs from 6 to 9 weeks old were selected from a commercial farm located in the same geographical area. One was a group of pigs diagnosed with meningitis (n = 11, six males and five females), and the other were clinically healthy pigs (n = 13, seven males and six females). The animals with meningitis had clinical signs compatible with this disease (ataxia, anorexia, lateral recumbency, and padding) [47] and were positive for the presence of Streptococcus suis in bacteriological cultures performed in blood agar plates following standard procedures [48]. Only saliva was obtained in this trial, aiming to avoid the stress associated with blood extraction.

The study protocol was approved by the Bioethical Commission of the University of Murcia, according to the European Council Directives regarding the protection of animals used for experimental purposes (CEEA 563/2019).

4.2. Sample Collection

Saliva was collected using a sponge clipped to a flexible thin metal rod of approximately 10 cm in length. Pigs were allowed to chew on the sponge until it was thoroughly moist. Then the sponges were removed from the pigs' mouths and placed in Salivette tubes (Sarstedt, Aktiengesellschaft & Co. D-51588 Nümbrecht, Germany). In all sampled pigs, saliva was first collected, and, after that, the animals were restrained with a nose sling to obtain a blood sample by the venipuncture of the jugular vein using vacuum plain tubes (BD Vacutainer, Franklin Lakes, NJ, USA). All samples were kept at 4–8 °C until arrival at the laboratory, where the vacutainer and the Salivette tubes were centrifuged at 3000 g and 4 °C for 10 min to obtain serum and saliva supernatant, respectively. Then, the aliquots were transferred into the Eppendorf tubes and stored at −80 ºC until the analysis was performed.

4.3. Sample Preparation for Proteomic Analysis

Proteomic analysis of saliva and serum samples was performed by TMT-based quantitative approach as described previously [49].

Briefly, saliva samples were centrifuged (13,000 g, 10 min, 4 °C), precipitated overnight in the ice-cold acetone, the supernatant resuspended in 1% SDS in 0.1 M triethyl ammonium bicarbonate (TEAB, Thermo Scientific, Rockford, IL, USA) buffer, and protein concentration was determined using the BCA assay (Thermo Scientific, Rockford, IL, USA). Furthermore, the preparation protocol was identical for the serum and saliva samples. An amount of 35 µg from the samples and internal standards (a pool of equal protein amounts from all samples) was reduced with dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA), alkylated with iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA), and precipitated overnight with ice-cold acetone (VWR, Radnor, PA, USA). Following the centrifugation (9000× g, 4 °C, 15 min), the protein pellets were dissolved in 0.1 M TEAB and digested using 1 mg/mL trypsin (Trypsin Gold, Promega; trypsin-to-protein ratio 1:35, at 37 °C overnight). TMT 6plex reagents (Thermo Scientific, Rockford, IL, USA) were prepared according to the manufacturer's procedure. An amount of 19 µL of the specific TMT label was added to each sample. After 60 min at the room temperature the addition of 5% hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA) quenched the reaction. Five TMT-modified samples were randomly combined with the internal standard, aliquoted, dried, and then liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis proceeded.

4.4. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

For LC-MS/MS analysis, we used the platform consisting of the Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) and the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Following their dissolution in the loading solvent (2% ACN, 0.1% formic acid), the labelled peptides were loaded onto the trap column (C18 PepMap100, 5 μm, 100A, 300 μm × 5 mm). After that, the separation on the analytical column (PepMap™ RSLC C18, 50 cm × 75 μm) followed. For achieving the separation gradient, we used two mobile phases, one was 0.1% formic acid in water (mobile phase A) and the other was 0.1% formic acid in 80% acetonitrile (mobile phase B). The separation protocol involved the linear gradient of 5–55% mobile phase B over 120 min, followed by 55% to 95% for 1 min, 95% for 2 min, and a decrease to 5% B during 20 min under the flow rate of 300 nL/min. The nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) with the 10 μm inner diameter SilicaTip emitter (New Objective, USA) was used for ionization. The MS operating parameters were a positive ion mode using DDA Top8 method, full-scan in range from m/z 350.0 to m/z 1800.0, resolution 70,000, 120 ms injection time, AGC target 1 × 106, isolation window ±2.0 Da, and the dynamic exclusion 30 s. The conditions for HCD fragmentation were step collision energy (29% and 35% NCE) with a resolution of 17,500 and AGC target of 2×105. The criteria to exclude the precursor ions from fragmentation were the unassigned charge state or the charge states +1 and higher than +7.

For protein identification and quantification, we followed the SEQUEST algorithm with the Proteome Discoverer software (version 2.3., ThermoFisher Scientific), searching against Sus scrofa FASTA files (downloaded from Uniprot database on December 2, 2020, 150,392 sequences). The identification parameters were: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification; oxidation (M); and TMT sixplex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) was set at 5%, as calculated with the Percolator algorithm in the Proteome Discoverer workflow. Only the confidently identified proteins (at least two unique peptides and 5% FDR) entered the bioinformatics analysis.

4.5. Bioinformatics

For Fold changes between groups were calculated as the log2(Mean(Group2)/Mean(Group1)). To test the significance of the difference in the relative abundance between the samples taken from the same group at the different time points, we applied the Friedman test with Dunn's multiple comparison test as the post-hoc analysis. Statistical significance was considered when $p < 0.05$. All statistical analyses were implemented using Python3 and the SciPy (Virtanen et al., 2020) and scikit-post hoc libraries.

Proteins were mapped to UniProt entries and annotated with the gene names, protein names, and descriptions. For the functional characterization of the differentially expressed proteins, Gene Ontology (GO) enrichment analysis was performed using the Cytoscape plugin ClueGo and its functionalities in order to fuse and group functionally related terms to reduce redundancy and REVIGO [50].

4.6. Validation Study

ALDOA was the protein selected for the validation study. The first step was the validation of the commercially available reagent kit (Aldolase, Randox Laboratories Ltd., Crumlin, UK) for the measurement ALDOA activity in saliva. This assay was applied to Beckman Coulter AU 400 autoanalyzer following the manufacturer's recommendations.

The validation was based on the following four features:

- Precision: The intra- and inter-assay coefficient of variation (CV) were calculated after analyzing two saliva samples of high and low concentration, respectively.

- Accuracy: The indirect evaluation by the linearity under the dilution of a saliva sample with a high ALDOA level.
- LLOQ: The lowest analyte concentration that could be measured with an intra-assay CV < 20%.
- LD: The lowest analyte concentration that could be distinguished from zero value. It was calculated based on data from ten replicate measurements of the zero standard (saline solution) as a mean value plus three standard deviations (SD).

Once validated, the assay was used to measure ALDOA in the saliva of LPS and turpentine pigs used in the proteomic study and for the comparison between pigs with meningitis and healthy pigs, as was indicated previously. The Friedman test followed by Dunn's multiple comparisons post hoc test was used to assess the significance between the three time points (basal, T6 h, and T24 h). In the case of pigs with meningitis and controls, the Mann–Whitney U test was used to compare salivary ALDOA activity. Additionally, the influence of gender in ALDOA measurements was investigated by a two-way ANOVA test. Results were expressed as median, and ranges and the difference were considered significant when the *p*-value was below 0.05.

5. Conclusions

The saliva and serum proteome of pigs showed changes in sepsis and also in non-septic inflammation in our experimental conditions. Aldolase A and serpin 12 were proteins in saliva that were significantly upregulated in sepsis and did not show significant changes in non-septic inflammation. In addition, GO analysis showed that different pathophysiological pathways in saliva were altered in pigs with sepsis compared with pigs with non-septic inflammation. In serum, increases in acute phase proteins were detected in both conditions and some proteins, such as APOE, showed significant changes in sepsis but not in non-septic inflammation.

Overall, these results indicate that proteins in saliva and serum can change, reflecting different pathophysiological mechanisms in sepsis and non-septic inflammation and some of these proteins could be potential biomarkers for these conditions. This should be considered as a pilot study since it included only male pigs in the proteomic study and the results were validated only in meningitis as a sepsis-related pathology but not in other infections.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/ijms23126738/s1.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethical Committee on Animal Experimentation (CEEA) of the University of Murcia (protocol code CEEA 563/2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Revealing the Changes in Saliva and Serum Proteins of Pigs with Meningitis Caused by Streptococcus Suis: A Proteomic Approach

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Abstract: Meningitis due to *Streptococcus suis* causes high mortality and morbidity on pig farms and has increasing zoonotic potential worldwide. Saliva proteome analysis would potentially be useful in elucidating pathophysiological changes and mining for new biomarkers to diagnose and monitor *S. suis* infection. The objective of this study was to investigate the changes in the salivary and serum proteome profile of piglets with meningitis. The LC-MS/MS TMT proteomic approach was used to analyze saliva and serum samples from 20 male piglets: 10 with meningitis and 10 healthy. In saliva, 11 proteins had higher and 10 had lower relative abundance in piglets with meningitis. The proteins with the highest relative abundance were metavinculin (VCL) and desmocollin-2 (DSC2). Adenosine deaminase (ADA) was selected for validation using a spectrophotometric assay and demonstrated excellent performance in the differentiation between healthy and pigs with meningitis due to *S. suis*. In serum, the most protruding changes occurred for one SERPIN and haptoglobin (HP). In saliva and serum, the highest number of proteins with altered abundance were linked, via the enrichment analysis, with platelet and neutrophil pathways. Overall, meningitis caused by *S. suis* resulted in specific proteome changes in saliva and serum, reflecting different pathophysiological mechanisms, and marking new potential biomarkers for this infection.

Keywords: *Streptococcus suis*; meningitis; pigs; saliva; proteomics; biomarkers

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1. Introduction

The *Streptococcus suis* is a Gram-positive bacteria considered one of the most important swine pathogens. It is associated with significant mortality and morbidity on pig farms, with an average of 14% post-weaning mortality in a previous study in Canada [1], being responsible for major economic losses in the swine industry [2]. In addition to meningitis, which is the common clinical manifestation, *S. suis* infection also can cause arthritis, pneumonia, or endocarditis [3]. It also has significant zoonotic potential, and infection in humans has similar clinical features as in pigs [4]. The number of cases in humans is increasing worldwide with cases of *S. suis* infections reported in more than 30 countries or regions of the world, mainly in Southeast Asia [5]. The rising incidence of human infections is associated with the increasing number of pig farms and the lack of preventive

measures to avoid the infection, such as consuming uncooked pig products or slaughtering practices without satisfactory preventive barriers [6].

Proteomics represents a comprehensive combination of analytical approaches endeavoring to decipher qualitative and quantitative protein composition in a sample [7]. Protein changes in the serum of piglets with an experimentally induced infection with *S. suis* were studied through a proteomic approach [8]. In this report, the authors highlighted ten serum proteins as potential biomarkers due to the different levels in the meningitis group compared with the control group. ADP ribosylation factor 4, immunoglobulin lambda-like polypeptide 5, phosphoglycerate mutase 1, and thioredoxin-1 were among the nine proteins with higher levels in the meningitis group, while complement component 4 binding protein alpha was the only protein with a lower level in piglets with the disease.

Saliva is a fluid attracting growing attention in the research of biomarkers for health and welfare evaluation. The easy sampling procedure, with minimal stress and no need for complex equipment, makes it convenient for large-scale sampling [9]. Proteomic studies using saliva have been published for the selected diseases in various species such as equine gastric ulcer syndrome in horses [10] or mastitis in cows [11] to gain knowledge about the pathophysiology of diseases and to find potential biomarkers. In pigs, a recent proteomic study explored the changes in saliva that occurs in sepsis induced by LPS administration [12]. In these studies, proteomic methodology relied on liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of peptides labelled with isobaric tandem mass tags (TMT), which allowed an accurate simultaneous relative quantification of multiple proteins [13].

However, to the best of our knowledge, no proteomic studies exploring the changes of protein content in the saliva of pigs with meningitis caused by *S. suis* have been performed. Thus

The main objective of this study was to investigate if the infection by *S. suis* produces changes in the salivary protein composition of piglets. Additionally, the serum proteomic profile was analyzed to compare with those changes observed in saliva.

2. Results

2.1. Salivary Proteomic Profile in Piglets with Meningitis

A total of 21 proteins showed different relative abundances between control and disease groups (Supplementary Table S1, Figures 1 and 2). The highest fold changes (FC) were: metavinculin (VCL), desmocollin 2 (DSC2), immunoglobulin heavy constant mu (IGHM), fructose-biphosphate aldolase (ALDOA) and a serpin domain-containing protein (SERPINB12). In the case of salivary proteins with lower relative abundance in MP, those with the most evident FC were lipocalin cytosolic FA-bd domain-containing protein (OBP2B), hemoglobin subunit beta (HBB), and double-headed protease inhibitor, submandibular gland-like (LOC100739218).

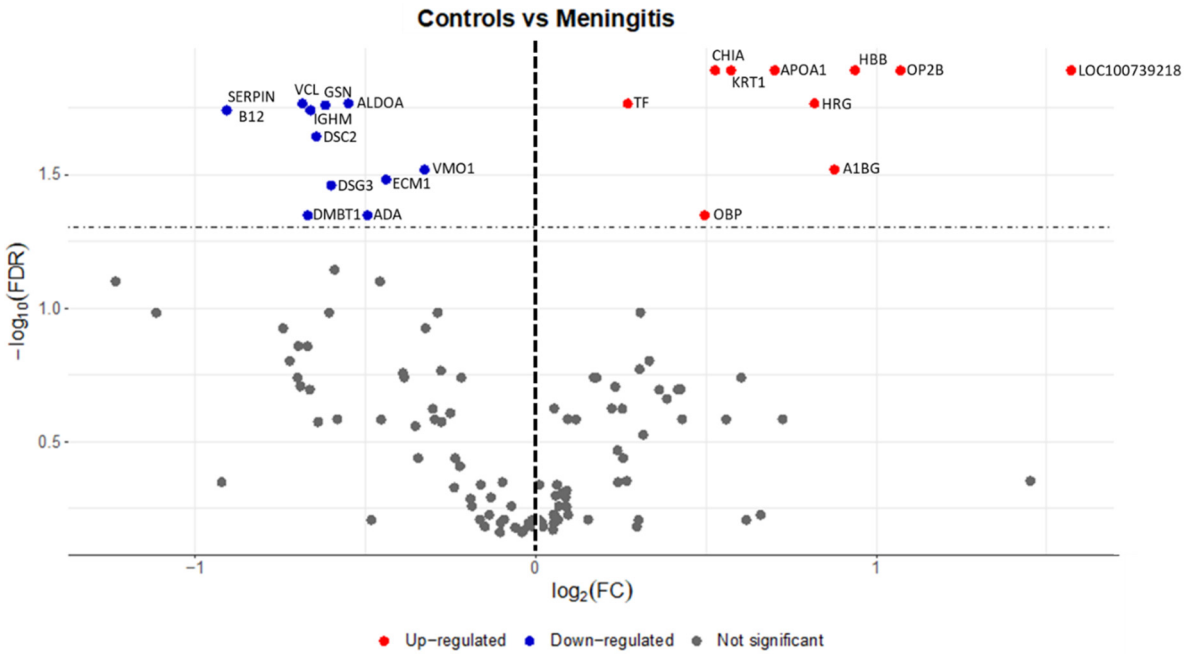

Figure 1. Volcano plot showing salivary proteins with the higher (red color) and lower (blue color) relative abundance between controls and pigs with meningitis. Gene symbols for the proteins are given on the right. Abbreviations: A1BG—Alpha-1B-glycoprotein, ADA—Adenosine aminohydrolase, ALDOA—Fructose-bisphosphate aldolase, APOA1—Apolipoprotein A-I, CHIA—Chitinase, DMBT1—Isoform 2 of Deleted in malignant brain tumors 1 protein, DSC2—Desmocollin 2, DSG3—Desmoglein 3, ECM1—Extracellular matrix protein 1, GSN—Actin-depolymerizing factor, HBB—Hemoglobin subunit beta, HRG—Cystatin domain-containing protein, IGHM—Immunoglobulin heavy constant mu, KRT1—Cytokeratin-1, LOC100739218—Double-headed protease inhibitor, submandibular gland-like, OBP—Odorant-binding protein, OBP2B—Lipocln_cytosolic_FA-bd_domdomain-containing protein, SERPINB12—SERPIN domain-containing protein, TF—Serotransferrin, VCL—Metavinculin, VMO1—Vitelline membrane outer layer protein 1.

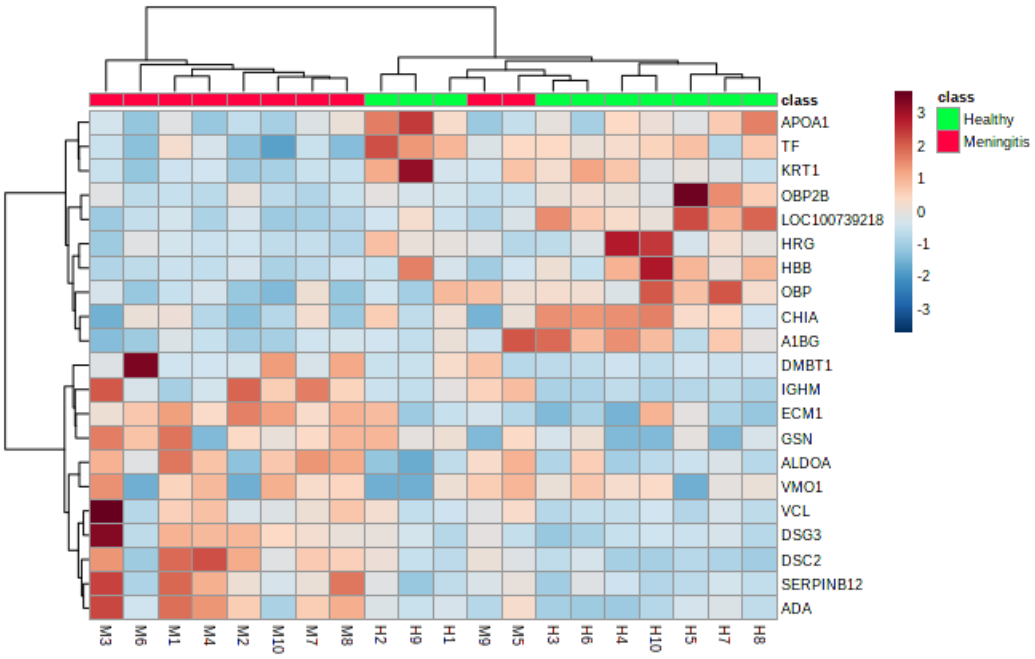

Figure 2. Hierarchical cluster analysis based on the proteins with the different relative abundance in saliva of the piglets with meningitis (red board) and the healthy piglets (green board). The red color represents the increased relative abundance, and the blue corresponds to the decreased relative abundance in meningitis versus the healthy group. Abbreviations: A1BG—Alpha-1B-

glycoprotein, ADA—Adenosine aminohydrolase, ALDOA—Fructose-bisphosphate aldolase, APOA1—Apolipoprotein A-I, CHIA—Chitinase, DMBT1—Isoform 2 of Deleted in malignant brain tumors 1 protein, DSC2—Desmocollin 2, DSG3—Desmoglein 3, ECM1—Extracellular matrix protein 1, GSN—Actin-depolymerizing factor, HBB—Hemoglobin subunit beta, HRG—Cystatin domain-containing protein, IGHM—Immunoglobulin heavy constant mu, KRT1—Cytokeratin-1, LOC100739218—Double-headed protease inhibitor, submandibular gland-like, OBP—Odorant-binding protein, OBP2B—Lipocln_cytosolic_FA-bd_domdomain-containing protein, SERPINB12—SERPIN domain-containing protein, TF—Serotransferrin, VCL—Metavinculin, VMO1—Vitelline membrane outer layer protein 1.

In addition, the PCA plot clearly indicated that samples formed separated clusters between groups (Figure 3).

The differentially expressed proteins in saliva between meningitis and healthy groups were used for analysis in terms of functional clusters, according to the PANTHER classification system (http://www.pantherdb.org, accessed on 20 July 2022) (Supplementary Table S2). The identified differentially abundant proteins had four molecular functions, with binding (GO:0005488) and catalytic activity (GO:0003824) as the most representative. Twelve biological processes were associated with these proteins, whereby the cellular process (GO:0009987) and metabolic process (GO:0008152) were those with the highest percentage of genes associated.

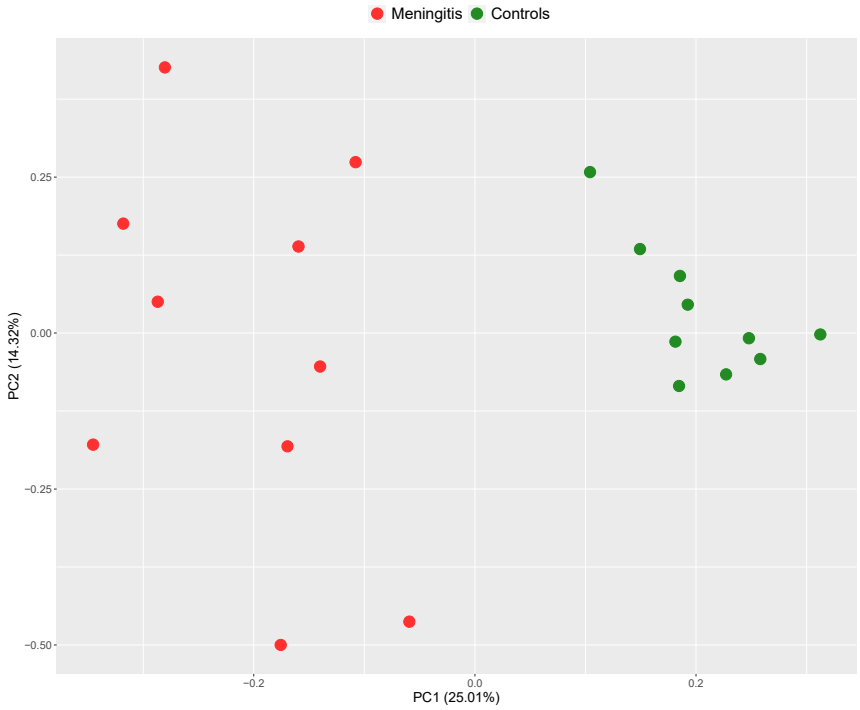

Figure 3. Principal component analysis (PCA) score plot of saliva samples showing the distribution of groups.

The pathway enrichment analysis showed alterations in nine different pathways, such as platelet degranulation, response to elevated platelet cytosolic Ca2+, platelet activation, signaling and aggregation, neutrophil degranulation, and innate immunity pathways (Table 1).

Table 1. Pathway enrichment analysis of the saliva proteins differentially abundant between the piglets with meningitis and healthy piglets. FDR < 0.05 is considered significant.

Pathway	FDR	Count		Genes
		Observed	Background	
Platelet degranulation	<0.001	7	141	TF, ECM1, APOA1, A1BG, ALDOA, HRG, VCL
Response to elevated platelet cytosolic Ca2+	<0.001	7	148	TF, ECM1, APOA1, A1BG, ALDOA, HRG, VCL
Hemostasis	<0.001	10	803	IGHM, TF, ECM1, HBB, APOA1, A1BG, ALDOA, HRG, VCL
Platelet activation, signaling and aggregation	<0.001	7	293	TF, ECM1, APOA1, A1BG, ALDOA, HRG, VCL
Neutrophil degranulation	<0.001	8	480	TF, SERPINB12, GSN, KRT1, HBB, A1BG, ALDOA, VCL
Amyloid fiber formation	0.010	3	89	TF, GSN, APOA1
Innate immune system	0.026	8	1345	TF, SERPINB12, GSN, KRT1, HBB, A1BG, ALDOA, VCL
Formation of the cornified envelope	0.027	3	138	KRT1, DSG3, DSC2
Apoptotic cleavage of cellular proteins	0.029	2	38	GSN, DSG3

FDR—False discovery rate (FDR).

2.2. Serum Proteomic Profile in Piglets with Meningitis

A total of 20 proteins were different in their relative abundances between the control and disease groups (Supplementary Table S3, Figures 4 and 5). Proteins with the highest abundance were three serpin domain-containing proteins (LOC106504547, LOC396684*, and LOC100156325), haptoglobin (HAPT), serum amyloid P-component (APCS), and lipopolysaccharide-binding protein (LBP). Within the serum proteins with lower abundance in the MP group, the most evident FC were observed in histidine-rich glycoprotein (HRG), apolipoprotein A-I (APOA1), and inter-alpha-trypsin inhibitor heavy chain H1 isoform a preproprotein (ITIH2).

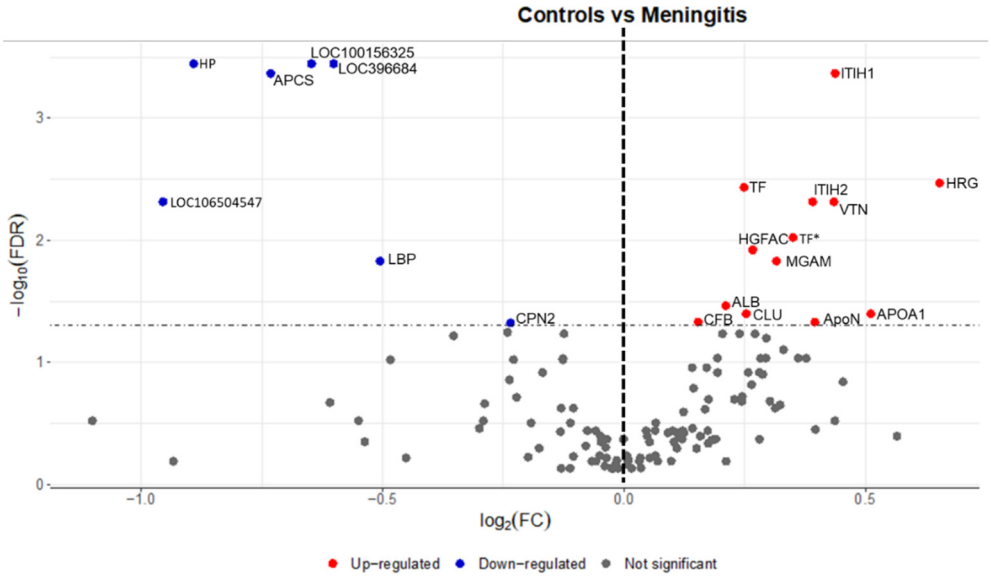

Figure 4. Volcano plot showing serum proteins with the higher (red color) and lower (blue color) relative abundance between controls and pigs with meningitis. Gene symbols for the proteins are given on the right. Abbreviations: *—identified after blasting in UniProt, ALB—Albumin

(Fragment), APCS—Serum amyloid P-component, APOA1—Apolipoprotein A-I, ApoN—Ovarian and testicular apolipoprotein N, CFB—C3/C5 convertase, CLU—Clusterin, CPN2—Carboxypeptidase N subunit 2, HGFAC—Hepatocyte growth factor activator isoform 2 preproprotein, HP—Haptoglobin, HRG—Histidine-rich glycoprotein, ITIH1—Inter-alpha-trypsin inhibitor heavy chain H1 isoform, ITIH2—Inter-alpha-trypsin inhibitor heavy chain H2, LBP—Lipopolysaccharide-binding protein, LOC100156325—SERPIN domain-containing protein, LOC106504547—SERPIN domain-containing protein, LOC396684—SERPIN domain-containing protein, MGAM—Maltase-glucoamylase (intestinal), TF—Serotransferrin/Beta-1 metal-binding globulin, VTN—Vitronectin.

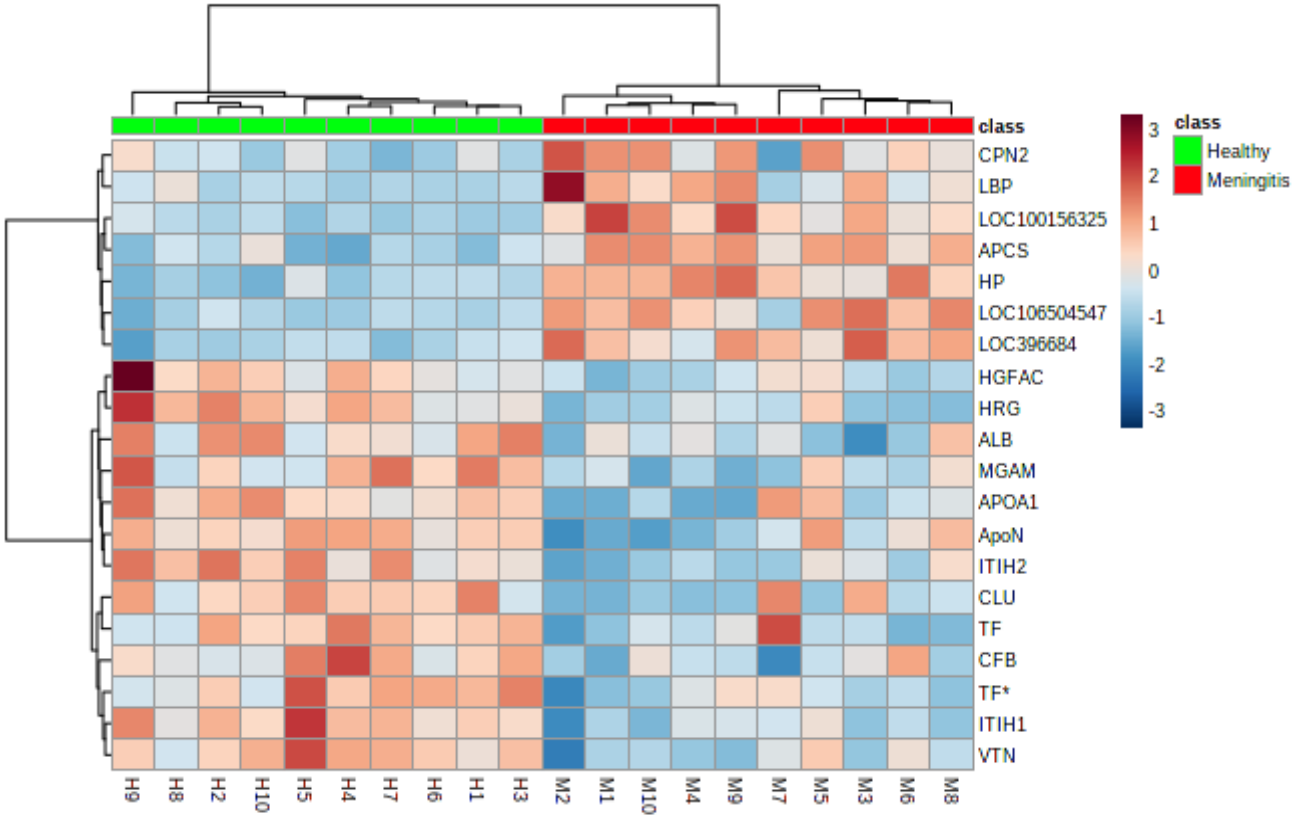

Figure 5. Hierarchical cluster analysis based on the proteins with the different relative abundance in serum of the piglets with meningitis (red board) and the healthy piglets (green board). The red color represents the increased relative abundance, and the blue corresponds to the decreased relative abundance in meningitis versus the healthy group. Abbreviations: *—identified after blasting in UniProt, ALB—Albumin (Fragment), APCS—Serum amyloid P-component, APOA1—Apolipoprotein A-I, ApoN—Ovarian and testicular apolipoprotein N, CFB—C3/C5 convertase, CLU—Clusterin, CPN2—Carboxypeptidase N subunit 2, HGFAC—Hepatocyte growth factor activator isoform 2 preproprotein, HP—Haptoglobin, HRG—Histidine-rich glycoprotein, ITIH1—Inter-alpha-trypsin inhibitor heavy chain H1 isoform, ITIH2—Inter-alpha-trypsin inhibitor heavy chain H2, LBP—Lipopolysaccharide-binding protein, LOC100156325—SERPIN domain-containing protein, LOC106504547—SERPIN domain-containing protein, LOC396684—SERPIN domain-containing protein, MGAM—Maltase-glucoamylase (intestinal), TF—Serotransferrin/Beta-1 metal-binding globulin, VTN—Vitronectin.

The PCA plot showed no signs of overlapping in the samples of each group; therefore, two different clusters were observed between the disease and control groups (Figure 6).

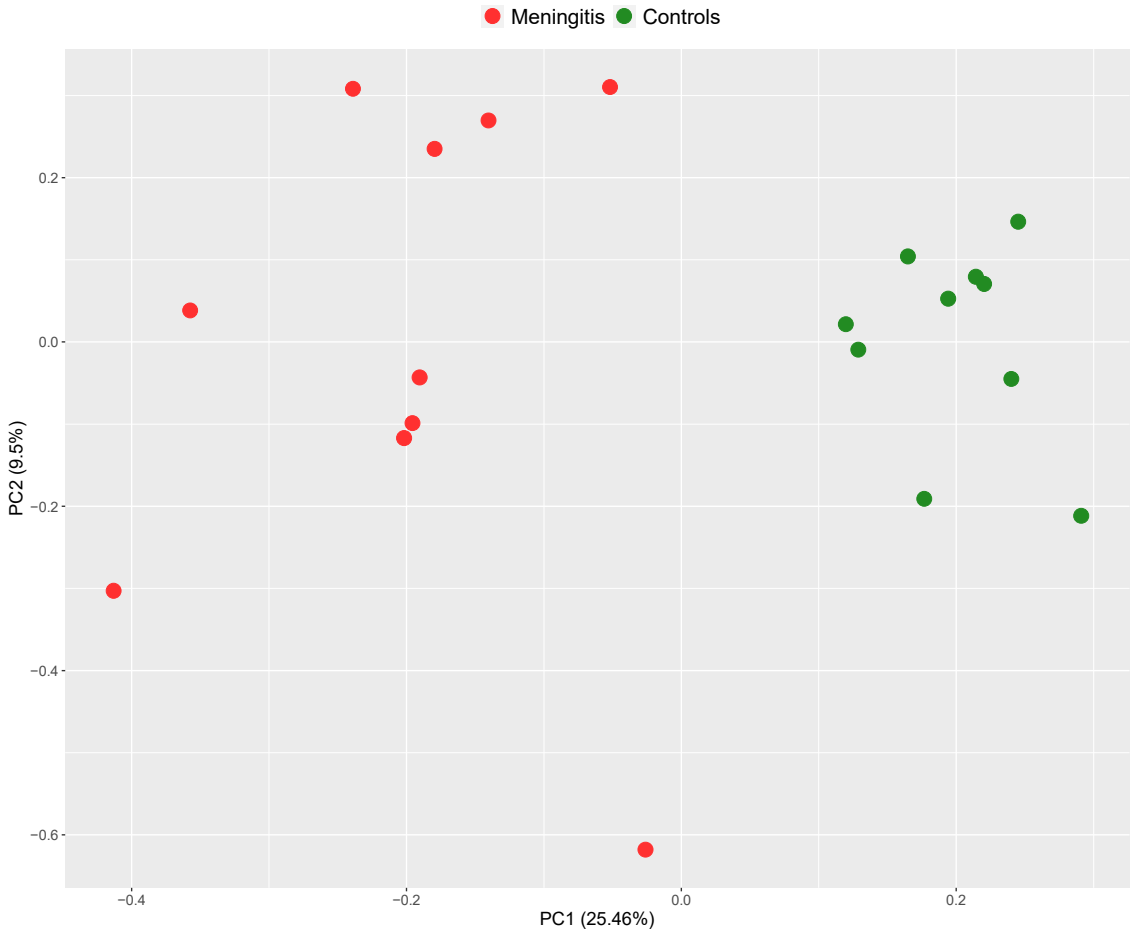

Figure 6. Principal component analysis (PCA) score plot of serum samples showing the distribution of groups.

The data from GO for the differentially abundant proteins in serum between pigs with meningitis and healthy pigs are reported in Supplementary Table S4. The identified differentially abundant proteins had three molecular functions, including binding (GO:0005488), catalytic activity (GO:0003824), and molecular function regulator (GO:0098772). Moreover, they participated in a total of 13 biological processes, with the cellular process (GO:0009987), response to stimulus (GO:0050896), and biological regulation (GO:0065007) reported as those with the higher percentage of genes associated.

Reactome pathway analysis of the statistically significantly different serum proteins between pigs with meningitis and healthy pigs showed alterations in 11 different pathways, whereby the most representative were those associated with platelet and neutrophil activation and complement system (Table 2).

Table 2. Pathway enrichment analysis of the serum proteins differentially abundant between the piglets with meningitis and healthy piglets. FDR < 0.05 is considered significant.

Pathway	FDR	Count		Genes
		Observed	Background	
Platelet degranulation	6.305×10^{5}	6	141	LOC100156325, TF, ALB, APOA1, HRG, CLU
Response to elevated platelet cytosolic Ca2+	1.859×10^{4}	6	148	LOC100156325, TF, ALB, APOA1, HRG, CLU
Post-translational protein phosphorylation	0.002	4	109	TF, ITIH2, ALB, APOA1
Complement cascade	0.002	5	156	APCS, VTN, CLU, CPN2, CFB

Regulation of complement cascade	0.017	4	139	VTN, CLU, CPN2, CFB
Regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding proteins (IGFBPs)	0.020	4	127	TF, ITIH2, ALB, APOA1
Neutrophil degranulation	0.020	5	480	LOC100156325, TF, MGAM, HP, LBP
Scavenging of heme from plasma	0.023	3	106	ALB, HP, APOA1
Platelet activation, signaling and aggregation	0.030	6	293	LOC100156325, TF, ALB, APOA1, HRG, CLU
Antimicrobial peptides	0.042	3	123	TF, LBP, CLU

2.3. Validation Study: Adenosine Deaminase (ADA) Activity of Saliva in Pigs with Meningitis

The measurements of salivary ADA activity showed significantly higher activity levels in pigs with meningitis caused by *S. suis* (median 12,480 U/L, minimum–maximum range 4928–35,360 U/L) compared with healthy pigs (median 1072 U/L, minimum–maximum range 281.6–3008 U/L) ($p < 0.001$) (Figure 7).

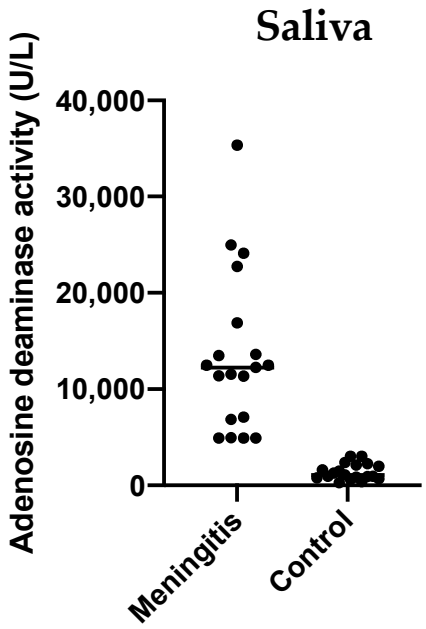

Figure 7. Adenosine deaminase (ADA) activity of saliva in the group OF meningitis compared with control. The plots show medians (line within box), 25th, and 75th percentiles (boxes), and min and max values (whiskers). Asterisks indicate statistically significant differences.

The ROC analysis indicated an excellent performance of ADA salivary activity with an area under the curve (95% confidence interval (CI)) of 0.983 (0.948–1.000). The calculated cut-off of 3106 U/L distinguished groups of piglets with meningitis and healthy piglets with equal sensitivity and specificity of 95% (75–100%).

3. Discussion

In this study, the changes in the salivary and serum proteome of pigs with meningitis caused by *S. suis* were evaluated. The high-resolution proteomic analysis showed a total of 21 salivary and 20 serum proteins changed in abundance in MP compared with HP.

3.1. Changes in Salivary Proteins in Pigs with Meningitis

In the case of saliva, of these 21 proteins, 11 were increased and 10 were decreased when pigs had meningitis. The molecular functions of these proteins were mainly related to binding capacity (40.7%) and catalytic activity (25.9%), and the most upregulated were VCL and DSC2.

VCL is a cytoskeletal protein considered a part of the complex that anchors actin to the cell membrane present among other tissues in the cardiac muscle [14]. Generally, vinculins play a key role in regulating cell adhesion, motility, and muscle endurance [15]. The presence of vinculins in muscle has been related to the contractile need of the cells; thus, the greater the contractile need of the muscle is, the greater the expression of the protein will be [16]. The increased expression of VCL in saliva could potentially be associated with muscle damage and seizures that usually are presented in the case of meningitis [14]. In this line, in our study, we have also observed an upregulation in the actin-depolymerizing factor (GSN) whose family is involved in the muscle actin filament organization and muscle contraction [17].

DSC2 is a cadherin that belongs to the desmocollins family. Molecules from the cadherin family are involved in the Ca2+-dependent mechanism for cell–cell adhesion [18], and DSC2 is only present in the desmosomes. The presence of DSC2 was related to cardiac alterations being associated with myocardial inflammation and fibrotic remodeling in mice [19] or in rhythm problems in humans [19,20]. The increased DSC2 in the saliva of pigs with meningitis could indicate possible myocardial damage since it is documented that cardiac injury is associated with the meningitis process in *S. suis* infections [21].

ADA was selected to validate the proteomic results in a larger population of pigs with meningitis due to the availability of an automated spectrophotometric assay validated for pigs [22]. ADA is a biomarker for inflammatory and immune disorders in pigs [23]. The increased ADA abundances in the saliva of pigs with meningitis could be due to the immune activation produced by the *S. suis* infection. This protein did not increase in the serum, confirming the different behavior in these two fluids that was previously documented [24]. Further studies should be conducted to explore the ability of ADA to detect non-sick carriers or to predict the outcome of the disease.

Although it was not specifically validated in our study, the increase found in ALDOA in the saliva is in agreement with a recent report in which increases in ALDOA were detected in the saliva of pigs with meningitis with a spectrophotometric assay [12] and in the serum of pigs with meningitis experimentally induced by *S. suis* [8].

Among the proteins with the lowest FC in the saliva of piglets with meningitis, OBP2B and HBB might be the most relevant. OBP2B belongs to the lipocalin family that is secreted by mandibular and submandibular glands in pigs [25]. Members of the lipocalin family like neutrophil gelatinase-associated lipocalin (NGAL) exerted a protective effect on the brain during inflammatory conditions in mice [26], and its deficiency results in a high susceptibility to worsening sepsis as was postulated previously [27].

HBB is a hemoglobin subunit, and this is the major heme protein of erythrocytes, facilitating the transport of oxygen and carbon dioxide in the blood [28]. An increase in serum HBB was considered an early predictor of sepsis in humans [29], and in pigs, serum HBB level increased in sepsis induced by lipopolysaccharide (LPS) administration [30]. However, we found a decreased expression of HBB in the saliva of pigs with meningitis, and a decreased level of this protein has been reported in the saliva of other animal species, like horses with acute abdominal disease [31]. Our data could be indicating a divergence in the behavior of HBB in saliva and serum in this condition that should be further explored.

The altered GO terms showed an intense activation of platelets during meningitis which is in line with previous data indicating the implication of platelets during sepsis [32]. In fact, it was reported the inhibition of platelet activation as a potential therapy in septic patients to prevent endothelial damage and organ failure [33]. This GO term would

indicate that platelets can be implied in the progress of the disease and the severity of its clinical manifestations in pigs.

As previously mentioned, some proteins that changed in pigs with meningitis, varied also in sepsis due to LPS injection, such as ALDOA, SERPINB12 or OBP2B [12]. However, in our study, there were specific proteins only showing changes in pigs with meningitis but not in pigs with sepsis due to LPS administration, like VCL, DSC2 or HBB. Further studies are needed to find proteins that could specifically and selectively change in this disease and differentiate it from other septic conditions.

3.2. Changes in Serum Proteins in Pigs with Meningitis

In serum, a total of 23 proteins were changed in their abundance in pigs with meningitis. In our study, the numbers of proteins with altered relative abundance were similar in saliva (21) and serum (23), but these proteins were different, with the exception of ALDOA, and serotransferrin (TF) which were downregulated in both fluids. However, the molecular function of the serum proteins that changed was associated with binding capacity (27.6%) and catalytic activity (27.6%) in a similar manner to saliva. The fact that different proteins were detected in saliva and serum may indicate that both biofluids would be providing complementary, although maybe not necessary correlative, information on the pathophysiology features of meningitis caused by *S. suis*. A similar observation occurred previously when saliva and serum were analyzed in horses with ulcers [10], cows with mastitis [11], or dogs with pyometra [34]. This highlights the hypothesis that saliva and serum could reflect different, but complementary pathophysiological features that occur in diseases [35].

Among the proteins that changed in the serum of pigs with meningitis, there were 7 upregulated and 13 downregulated. The most upregulated proteins were a serpin domain-containing protein (LOC106504547), HAPT, and APCS. Within the downregulated proteins, the most protruding changes were observed in histidine-rich glycoprotein (HRG), and apolipoprotein A-I (APOA1).

Three SERPINs different from the SERPINB12, found in saliva, had increased relative abundance in serum of pigs with meningitis (LOC106504547, LOC396684, and LOC100156325). These increases could be related to the protective role of these proteins. Two of these serpins (LOC106504547, and LOC396684) were previously reported upregulated in the serum of pigs with sepsis [12] and therefore could be associated with septic conditions.

HP is a plasma protein that tightly captures hemoglobin (Hb) during hemolysis [36]. It is considered a moderate acute phase protein in swine [37,38]. The concentration of haptoglobin has been previously described as increased in inflammatory and infectious processes such as the administration of LPS [12,30,39,40], and viral infections [41].

APCS is a glycoprotein that belongs to the family of pentraxins closely related to C-reactive protein (CRP) [42] that regulates several aspects of the innate immune system such as the inhibition of fibrocytes and neutrophil adhesion to extracellular matrix proteins or promoting the immuno-regulatory macrophages [43]. The main features of APCS lie in the modulation of the humoral innate immune system spanning the complement system, inflammation, and coagulation [44]. In line with these data are the pathway enrichment analysis results indicating APCS as one of the main proteins associated with the complement cascade pathway.

Within the downregulated proteins, the most protruding changes were observed in histidine-rich glycoprotein (HRG), and apolipoprotein A-I (APOA1).

HRG is a 75 kDa glycoprotein synthesized in the liver and released into the bloodstream to modulate sepsis-related biological reactions by binding to several substances (like heparin, factor XII, fibrinogen, thrombospondin, IgG, C1q, among others) and cells [45]. The HRG was also observed downregulated in the serum of pigs with sepsis in a previous study [12]. We also showed the implication of HRG in the platelet degranulation pathway. The decreased abundances of serum HRG may lead to a hypercoagulative state,

fibrinolysis, and enhanced immune response that have been reported before in sepsis [46]. Additionally, HRG could be of particular importance in bacterial infection due to its ability to bind these pathogens [47]. Thus, the reduction of HRG might suggest a depleted capacity of the organisms to "fight" against bacterial infection and a predisposition to complications associated with it.

APOA1 is a negative acute phase protein and the major protein of high-density lipoprotein (HDL). Previous studies reported the anti-inflammatory and antithrombotic properties inherent to this protein, due to its ability to reduce the production of proinflammatory cytokines and chemokines [48]. APOA1 was also found down-regulated in the serum of pigs with sepsis induced by LPS injection [12], and its reduction in serum was documented after an experimental infection by *S. suis* [49]. APOA1 is considered a good predictor of infectious disease in pigs, due to levels dropping rapidly when the infection appears [50]. In the same manner that occurred with HRG, the relation observed with the platelet degranulation pathway may suggest that APOA1 could be a potential indicator of the presence of possible complications or increased severity in sepsis. However, this application needs to be validated in a future study when the progression of sepsis would be assessed.

In a previous publication, the serum proteome of pigs with meningitis caused *by S. suis* was studied and they reported up to 316 differentially expressed proteins in the disease group [8]. The higher number of proteins observed could be due to the different methods used since they studied meningitis in an experimental-induced model of pigs. Interestingly, we found two proteins that matched with that encountered by this article: ALDOA and HRG. In our case, ALDOA was detected in saliva, being a possible useful marker for sepsis condition. HRG was decreased in the serum of our pigs with meningitis, but it was increased in experimentally induced meningitis [8]. Further studies would be necessary to elucidate the reason for the different behaviour of HRG in both studies. For example, a possible cause for this could be the different breeds used in both reports since the previous publication used Bama miniature pigs, while our study was made with Large White pigs.

3.3. Clinical Implications of Differentially Expressed Salivary Proteins in Pigs with Meningitis

The expression of different proteins in saliva could be related to the clinical alterations manifested by the pigs affected by meningitis. For instance, the increased expression of VCL is an indicator of muscle damage and seizures, while DSC2 showed more specificity for myocardial damage during *S. suis* infection [21]. Therefore, further studies could be performed to evaluate if the magnitude of the increase of these proteins could be associated with the presence of more severe clinical signs and also if the decrease of these proteins after treatment could predict improvement in the clinical sign of the pigs.

This study has some limitations that should be considered. First, the small population of pigs used in the proteomic study creates the need to confirm these findings in a larger population. Moreover, only male pigs were used in this study and sex differences have not been assessed in the proteomic study, so possible effects of gender should be considered. We preferred to only use animals of one sex in this report in order to avoid bias due to the influence of hormonal effects. In addition, future studies should address if haematology and serum biochemistry data could be correlated to proteomic findings. In this study, we investigated the presence of meningitis due to *S. suis,* but since meningitis can be multicausal (including non-septic causes), comparative studies using pigs with meningitis due to other septic and non-septic causes should be performed to contrast the relevance of proteins found in our study.

4. Materials and Methods

4.1. Animals

For the proteomic study, we used 20 male weaning pigs [(Sus scrofa domesticus) (Large White)], from a commercial farm located in the region of Murcia (Spain). The piglets were from 6 to 9 weeks old. They were divided into two groups: (1) the control group, consisting of clinically healthy pigs (HP, n = 10), and (2) the disease group, consisting of pigs diagnosed with meningitis due to *S. suis* (MP, n = 10). Allanimals in the disease group presented clinical symptomatology (ataxia, anorexia, lateral recumbency, and padding) [51] that raised suspicion of meningitis [32] and were positive to *S. suis* as described in point 4.3. No prior potential animal exclusion criteria were established.

For the validation of proteomic results, additional groups were included, consisting of 19 male pigs between 6 to 9 weeks old, diagnosed with *S. suis*-associated meningitis, and 19 healthy male pigs of the same age, sampled by the same approach that was used for the proteomic study.

All procedures were approved by the Ethical Committee on Animal Experimentation (CEEA) of the University of Murcia (protocol code CEEA 563/2019).

4.2. Saliva and Serum Collection

Paired saliva and serum were taken from pigs included in the study. Saliva was collected using a sponge clipped to a flexible thin metal rod approximately 20 cm in length. Pigs had thoroughly moistened the sponge by chewing, and then, the sponges were placed into Salivette® tubes (Sarstedt, Aktiengesellschaft and Co. Nümbrecht, Germany).

After saliva collection, blood samples were obtained by puncturing the jugular vein and collected into vacuum plain tubes (BD Vacutainer, Franklin Lakes, NJ, USA).

All samples were kept at 4–8 °C in a portable refrigerator until arrival at the laboratory (in the next 15 min), where the vacutainer and the Salivette tubes were centrifuged at 3000× *g* and 4 °C for 10 min to obtain serum and saliva supernatant. Then, the aliquots were transferred into the Eppendorf tubes and stored at −80 °C until the analysis.

4.3. Streptococcus suis Isolation and Typification

Bacterial isolation and characterization were performed on the blood samples from clinically ill piglets. Samples were incubated on Columbia blood agar plates (Oxoid Ltd., Madrid, Spain) containing 5% defibrinated pig blood after 48 h at 37 ºC under aerobic conditions, as previously reported [52]. The isolates were identified following standard procedures and confirmed by a PCR based on the glutamate dehydrogenase gene [53].

All animals of the meningitis group tested positive for *S. suis* serotype 9.

4.4. Proteomic Analysis

Proteomic analysis of saliva and serum samples was performed by a TMT-based quantitative approach as described previously [54].

Briefly, saliva samples were centrifuged (13,000× *g*, 10 min, 4 °C), and the proteins precipitated overnight by ice-cold acetone (VWR, Radnor, PA, USA). The pellet was resuspended in 1% SDS in 0.1 M triethyl ammonium bicarbonate (TEAB, Thermo Scientific, Rockford, IL, USA). The total protein concentrations in saliva and serum were measured with the commercial bicinchoninic acid-based reagent (Thermo Scientific, Rockford, IL, USA). The amount of 35 μg of the samples (saliva or serum) and internal standards (a pool of equal protein amount from all saliva or serum samples) were reduced using dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA), followed by alkylation with iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA), and overnight precipitation with ice-cold acetone. The protein pellets were collected via centrifugation (9000× *g*, 4 °C, 15 min) and dissolved in 0.1 M TEAB prior to digestion with trypsin (Trypsin Gold, Promega; 1 mg/mL; trypsin-to-protein ratio 1:35, at 37 °C overnight). The next step was TMT (Thermo Scientific, Rockford, IL, USA) labelling, according to the manufacturer's instructions. The

19 µL of the specific TMT label was mixed with each sample. Following the labelling for 60 min at room temperature, the reaction was stopped with 5% hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA). A mixture of the five randomly chosen TMT-labeled samples and the internal standard was prepared for LC-MS/MS analysis.

The modular system integrating the Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) and the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used for LC-MS/MS analysis. Following their dissolution in the loading solvent (2% ACN, 0.1% formic acid in water), the labeled peptides were loaded onto the trap column (C18 PepMap100, 5 µm, 100A, 300 µm × 5 mm). After that, the separation on the analytical column (PepMap™ RSLC C18, 50 cm × 75 µm) followed. For achieving the separation gradient, we used two mobile phases, mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in 80% acetonitrile). The separation of the proteins was achieved by using the linear gradient of 5–55% mobile phase B over 120 min, followed by 55% to 95% for 1 min, 2 min at 95%, and decrease to 5% B during 20 min under the flow rate of 300 nL/min. The nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) with the 10 µm-inner diameters SilicaTip emitter (New Objective, Littleton, MA, USA) was used for ionization. The MS was operated as follows: positive ion mode using the DDA Top8 method, full scan in the range from m/z 350.0 to m/z 1800.0, resolution 70,000, 120 ms injection time, AGC target 1×10^6, isolation window ± 2.0 Da, and the dynamic exclusion 30 s. The conditions for HCD fragmentation were stepped collision energy (29% and 35% NCE) with a resolution of 17,500 and an AGC target of 2×10^5. The criteria to exclude the precursor ions from fragmentation were the unassigned charge state, or the charge states +1 and higher than +7 were excluded. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD037430.

For protein identification and quantification, we employed the SEQUEST algorithm with the Proteome Discoverer software (version 2.3., ThermoFisher Scientific, Waltham, MA, USA), searching against Sus scrofa FASTA files (downloaded from Uniprot database on 2 December 2020, 150,392 sequences). The identification parameters were: two trypsin missed cleavage sites, precursor, and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M), and TMT six-plex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) was set at 5%, as calculated with the Percolator algorithm in the Proteome Discoverer workflow. Only the confidently identified proteins (at least two unique peptides and 5% FDR) entered the bioinformatics analysis.

4.5. Statistics and Bioinformatics Analyses

Statistical analyses of proteomics results were performed using R software v.4.1.2. [55], following a previously published protocol [56]. In brief, sample outliers were detected by Dixon's test from R package outliers v0.14 and excluded from the further analysis, and the difference in protein abundance between MP and HP was accessed by the Mann–Whitney test. The *p*-values were adjusted using the false discovery rate (FDR) from R package qvalue v2.2.2, and differences with FDR < 0.05 were considered significant. Protein abundance fold changes between two groups were calculated as median (Group MP)/median (Group HP) and expressed on the log2 scale. Principal component analysis (PCA) and volcano plots were designed using the R package ggplot2 v3.1.1.

Further, the hierarchical clustering was analyzed for the proteins with the different relative abundance in saliva or serum between the piglets with meningitis and healthy piglets. The individual data were entered into the software (MetaboAnalyst™ version 5.0) without any transformation, and the clustering was based on the Euclidean distance.

The Protein Analysis Through Evolutionary Relationship (PANTHER) tool (http://www.pantherdb.org/, access date on 20 July 2022) with the subset of GO terms (GO Slim database) was employed for functional enrichment analyses [57]. The REACTOME tool, using the human genome as the background, was accessed for the pathway

enrichment analysis [58]. Significantly enriched pathways were considered for those with FDR-adjusted *p*-value < 0.05.

4.6. Validation Study

Among the proteins identified with the relative abundance in saliva differing between MP and HP, ADA was selected as a biomarker candidate for validation in an additional group of pigs with *S. suis*-associated meningitis (n =19) which was compared with a group of healthy pigs (n =19).

The activity of ADA was measured using an automated assay that was previously validated in the saliva of pigs [59]. Salivary ADA activity between pigs with meningitis and healthy pigs was compared using the Mann–Whitney U. Results were expressed as median and interquartile ranges, and the differences were considered significant when the *p*-value was below 0.05. Further, diagnostic accuracy was assessed via the Receiver Operating Characteristic (ROC) curve analysis, and the cut-off value, with optimal sensitivity and specificity, was determined. In the validation study, MedCalc™ software (version 16.2.1) was used for statistical analyses.

5. Conclusions

The analysis of the salivary and serum proteome of pigs with meningitis produced by *S. suis* evidence that this disease produced changes in proteins in both fluids. A similar number of proteins was changed in both saliva and serum, but their nature was different. VCL and DSC2 were the most upregulated proteins in saliva, both being related to muscle damage. The upregulation of ADA would reflect the immune system activation associated with sepsis. In serum, the proteins showed an upregulation of a serpin protein reflecting a protective response of the organism and an increase in HAPT indicating inflammation. Overall, the proteins changed to reflect the physiological mechanisms associated with meningitis in pigs caused by *S. suis* and could be potential biomarkers of this disease.

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Article 4 (published):

Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by Escherichia coli

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Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by *Escherichia coli*

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Abstract: *Escherichia coli* represents the main cause of diarrhoea in pigs. Saliva can provide information about the pathophysiology of diseases and be a source of biomarkers. We aimed to identify changes in the salivary proteome of pigs with diarrhoea caused by *E. coli*. Saliva samples were collected from 10 pigs with this disease and 10 matched healthy controls. SDS-PAGE (1DE) and two-dimensional gel electrophoresis (2DE) were performed, and significantly different protein bands and spots were identified by mass spectrometry. For validation, adenosine deaminase (ADA) was measured in 28 healthy and 28 diseased pigs. In 1DE, increases in lipocalin and IgA bands were observed for diseased pigs, whereas bands containing proteins such as odorant-binding protein and/or prolactin-inducible protein presented decreased concentrations. Two-dimensional gel electrophoresis (2DE) results showed that saliva from *E. coli* animals presented higher expression levels of lipocalin, ADA, IgA and albumin peptides, being ADA activity increased in the diseased pigs in the validation study. Spots containing alpha-amylase, carbonic anhydrase VI, and whole albumin were decreased in diseased animals. Overall, pigs with diarrhoea caused by *E. coli* have changes in proteins in their saliva related to various pathophysiological mechanisms such as inflammation and immune function and could potentially be biomarkers of this disease.

Keywords: *E. coli*; salivary proteome; pigs; diarrhoea; lipocalin; ADA; biomarkers

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1. Introduction

Nowadays, saliva is considered an innovative and important source of biomarkers for many diseases in animals and humans. Overall, its composition can change due to stress, inflammation and alterations in the immune system or redox status, which can lead to the use of saliva analytes as biomarkers of pathological conditions [1]. This type of biological sample collection has many advantages, as it is painless and can be obtained by easy and non-invasive methods. In fact, saliva can be sampled without the need for specialized personnel in the field, anytime and anywhere [2]. Saliva is especially valuable in pigs, as in this species the collection of blood is stressful and painful for the animals [2].

It has been observed that saliva can show proteomic changes in sepsis experimentally induced by lipopolysaccharide (LPS) administration in pigs [3]. Aldolase A and serpin 12 were proteins in saliva that were significantly upregulated in sepsis. In addition, the proteome of saliva in pigs with *Streptococcus suis* infection has been studied, with the proteins metavinculin (VCL) and desmocollin-2 (DSC2) showing the highest relative abundance [4].

Moreover, proteomic changes have been reported in the saliva of pigs in situations of compromised welfare, with the proteins cornulin, heat shock protein 27, and lactate dehydrogenase (LDH) showing significant increases, and the immunoglobulin J chain showed a significant decrease [5].

Enterotoxigenic Escherichia coli (ETEC) is considered one of the main causes of diarrhoea in piglets [6], having a major economic impact on swine production [7]. ETEC produces several virulence factors, such as colonization factors (adhesins) and/or toxins. Colonization factors promote adherence to the host small intestine, and enterotoxins stimulate the lining of the intestine and induce watery diarrhoea [6], leading to sepsis [8]. Proteomic studies have been made to evaluate the changes in the intestine of pigs with *E. coli* diarrhoea [6,7] but, to our knowledge, no studies have been made in saliva.

The main objective of this study was to evaluate the possible changes in the salivary proteome of pigs with diarrhoea caused by *E. coli*, compared to healthy controls. To this end, SDS-PAGE and 2DE gel electrophoresis were used for the separation of proteins. After profile comparison, the mass spectrometry technique was used for the identification of the proteins differentially expressed between diseased and healthy animals. In addition, one protein showing significant changes in the proteomic study was selected for validation.

2. Materials and Methods

2.1. Population of Animals

For the proteomic studies, two groups of Large White weaning pigs from 6 to 9 weeks old were selected from commercial farms located in Southern Spain. One was a group of pigs diagnosed with diarrhoea caused by *E. coli* ($n = 10$, half males and half females), and the other were clinically healthy pigs ($n = 10$, half males and half females). The diseased animals had clinical signs compatible with this disease (diarrheic syndrome) and were positive for the presence of *E. coli* in rectal swabs following standard analytical procedures [9], being positive for *E. coli* F4 and heat-labile toxin. Additionally, 28 healthy pigs and 28 pigs with diarrhoea caused by *E. coli* from 6 to 9 weeks old were used for the validation study.

2.2. Saliva Collection and Sample Processing

A sponge was used for saliva collection. The pigs were allowed to chew on the sponge until it was thoroughly moist. Then, the sponges were placed in Salivette tubes (Sarstedt, Aktiengesellschaft & Co., D-51588 Nümbrecht, Germany) and kept at 4−8 °C until arrival at the laboratory, where the Salivette tubes were centrifuged at 3000× *g* and 4 °C for 10 min to obtain saliva supernatant. Saliva was transferred into the Eppendorf tubes and stored at −80 °C.

2.3. SDS PAGE

This technique was made according to a previously published procedure [10]. Proteins from individual saliva samples from all young animals (both healthy and diseased) were separated by SDS-PAGE gel electrophoresis on 12% acrylamide gels using Bio-Rad equipment (mini-protean, Bio-Rad, Alges, Portugal). Samples were carried out in duplicate to minimize technical errors. The total protein concentration of the samples was determined using the BCA assay (Thermo Scientific, Rockford, IL, USA). Briefly, a total of 9 µg of protein from each saliva sample was lyophilised and reconstituted with 40 µL of sample buffer (62.5 mM Tris-HCl pH 6.8, 2% (*w*/*v*) SDS, 10% glycerol, 5% DTT and bromophenol blue). Then, the samples were placed on ice and heated for 5 min at 98 °C to denature proteins. The Bio-Rad electrophoresis tank system was set up with running buffer (0.025 M Tris HCl, 0.192 M Glycine, and 0.1% (*w*/*v*) SDS; pH 8.3. Twenty µL of the reconstituted sample were applied to each lane (in duplicate), and electrophoresis was run at a constant voltage of 150 V until the dye front reached the end of the gel. The gels were fixed in 40% methanol, and 10% acetic acid for one hour, stained with Coomassie Brilliant Blue R-250 (0.2% in 40% methanol, 10% acetic acid) for another hour, and destained with 10% acetic acid several times until staining background remotion. Finally, LabScan software was used to acquire

scanned images of the gels, and ImageLab software (Bio-Rad, Alges, Portugal) was used for gel analysis.

2.4. Two-Dimensional (2-DE) Gel Electrophoresis

For the 2DE technique, 3 pools of pig saliva samples were prepared from the group of healthy pigs and other 3 pools from the group of pigs with diarrhoea caused by *E. coli*. The volume of each individual corresponded to the same amount of total protein, in order to have a final total volume corresponding to 275 µg of total protein (determined using the BCA assay (Thermo Scientific, Rockford, IL, USA). Each pool was lyophilized and stored at −28 °C. The solid material was reconstituted with 250 µL of solubilization buffer [7 M urea, 2 M thiourea, 4% (*w*/*v*) 3-(3-cholamidopropyl) dimethylammonium propane sulfonate (CHAPS), 2% (*v*/*v*) ampholyte mixture (IPG buffer pH 3-11, GE Healthcare, Chicago, IL, USA), and 40 mM dithiothreitol (DTT)]. The mixture was incubated for 1 h at room temperature and subsequently centrifuged for 10 min at 10,000 rpm at room temperature. After this, the supernatant from each sample was divided into two volumes of 125 µL and applied in two different slots of the strip holder of the Multiphor II system (GE Healthcare, Chicago, IL, USA) to run each sample in duplicate. The last step in strip rehydration was to place the commercial gel strips [7 cm pH gradient 3–11 NL (IPG strips, GE Healthcare, Chicago, IL, USA)] in contact with the sample and leave them in passive rehydration overnight at room temperature, covered with mineral oil. Focusing was performed in a Multiphor II (GE, Healthcare, Chicago, IL, USA) at 12 °C with the following program (gradient): (1) 0–150 V for 15 min; (2) 150–300 V for 15 min; 300 V for 0.5 h; 300–3500 V for 4 h; 3500 for 3.5 h. Focused strips were equilibrated and applied on top of a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel and run at 150 V constant voltage on a mini-protein system (Bio-Rad, Alges, Portugal). Staining was made with CBB-R250. The image acquisition of the gels was made by a gel scanner (ImageScanner III, GE Healthcare, Chicago, IL, USA) and Lab scan software (GE Healthcare, Chicago, IL, USA), and the analysis was performed using the SameSpots software (v5.1.012, TotalLab, Gosforth, UK).

2.5. In-Gel Trypsin Digestion

After image analysis, the bands and spots that were observed to differ, in relative amounts, between healthy and *E. coli* individuals in SDS-PAGE and 2DE gels were selected for identification by MS. They were spliced into approximately 2 × 2 mm parts and distained. Then, they were alkylated and incubated with trypsin (Promega Corporation, Madison, MI, USA) and ProteaseMax surfactant (Promega Corporation, Madison, MI, USA) for 10 min at 4 °C. Finally, samples were digested at 37 °C for 16 h.

2.6. Protein Identification through HPLC-MS/MS Analysis

An HPLC/MS system consisting of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) connected to an Agilent 6550 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used in this study. Parameters for the equipment analysis were set in MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00, Santa Clara, CA, USA).

Dry samples from trypsin digestion were resuspended in a buffer with water/acetonitrile/formic acid and injected onto an Agilent AdvanceBio Peptide Mapping HPLC column, thermostated at 50 °C, at a flow rate of 0.4 mL/min.

The data processing and protein identification was made on Spectrum Mill MS Proteomics Workbench (Rev B.06.00.201, Agilent Technologies, Santa Clara, CA, USA). The criteria used for MS/MS search against the appropriate and updated protein database were: variable modifications search mode (carbamidomethylated cysteines, STY phosphorylation, oxidized methionine, and N-terminal glutamine conversion to pyroglutamic acid); tryptic digestion with 5 maximum missed cleavages; ESI-Q-TOF instrument (Agilent Technologies, Santa Clara, CA, USA); minimum matched peak intensity 50%; maximum ambiguous pre-

cursor charge +5; monoisotopic masses; peptide precursor mass tolerance 20 ppm; product ion mass tolerance 50 ppm; and calculation of reversed database scores.

2.7. Statistical Analysis

The data were evaluated for normal distribution using the Shapiro–Wilk test. Variables (protein concentration, protein bands and spots) for which normal distribution was not observed were transformed (log transformation). When normal distribution was achieved, Student's *t*-test was used for group comparison, whereas non-normally distributed variables were compared using a non-parametric test (Mann–Whitney). Statistical analysis was performed with SPSS (v.28.0, IBM SPSS Statistics, New York, NY, USA). Statistically significant differences were considered when the p-value < 0.05.

2.8. Validation

Among the proteins identified with the relative abundance in saliva showing significant changes between healthy and diseased pigs, ADA was selected as a biomarker candidate for validation in an additional group of pigs with *E. coli* diarrhoea (n = 28), which was compared with a group of healthy pigs (n = 28). In both groups, half of the animals were male and half female.

The activity of ADA was measured using an automated assay that was previously validated in the saliva of pigs [11].

3. Results

3.1. Total Protein Concentration

The total protein concentration of saliva samples was observed to be significantly higher in *E. coli*-diseased animals compared to the healthy ones. Mean *E. coli* animals have almost 3 times higher values of total protein than healthy animals (76.4 ± 41.8 µg/mL vs. 280.5 ± 107.7 µg/mL, for healthy and *E. coli* groups, respectively; p = 0.001).

3.2. SDS-PAGE Profile

Salivary SDS-PAGE protein profiles allowed the constant visualization of clearly distinct 21 protein bands, with molecular masses between 10 and 200 kDa, whose levels were compared between groups (Figure 1).

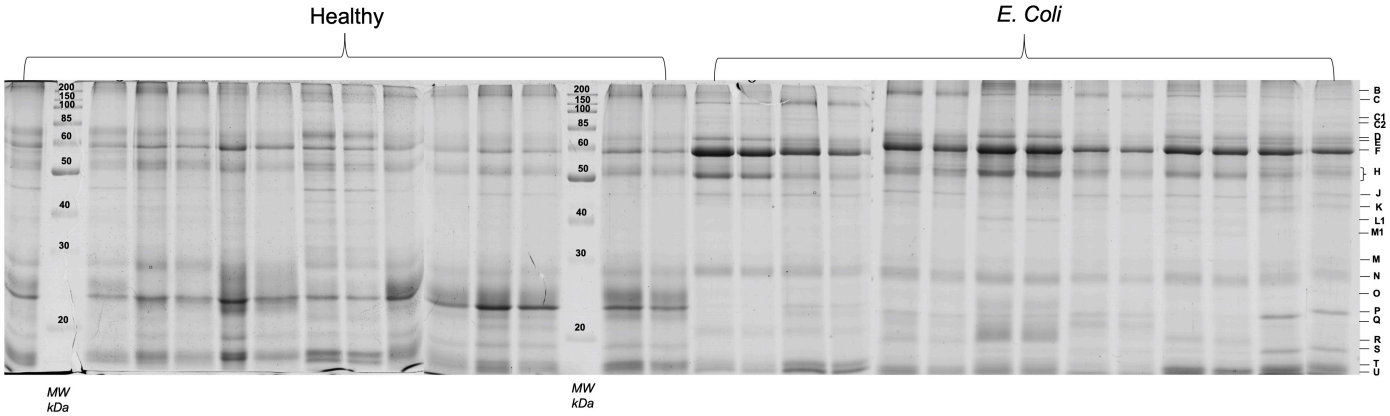

Figure 1. Salivary protein profiles (SDS-PAGE) of all the samples (healthy controls and *E. coli* diseased pigs). Each capital letter, on the right side, represents the bands compared between groups.

Eight protein bands were observed to be differently expressed between healthy and diseased animals. Band C1 was a faint band, not identified through mass spectrometry, which was only observed in the *E. coli* group. The other 7 bands, although observed in animals from both groups, presented statistically significant differences, with bands B, H, M, N, and R increasing in diseased animals and bands P and T decreasing in those. The

differences between groups, as well as mass spectrometry identifications of the proteins present in those bands, are presented in Table 1.

Table 1. Differences in protein band expression levels (mean ± standard deviation of %Vol) between *E. coli* diseased and healthy pigs and correspondent protein identification and MS.

Band	Healthy	*E. coli*	*p*-Value	UNIPROT Protein Accession Number	Protein (Entry Name)	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
B	1.62 ± 0.80	5.36 ± 3.06	0.001	018758	Submaxillary apomucin	1.3	238.4	1184.1	>200 kDa
C1	-				ni				120
H	3.74 ± 0.59	9.77 ± 2.91	0.0005	A0A287B626	IgA constant region	39.3	209.6	44.2	54
M #	1.35 ± 1.09	2.94 ± 0.75	0.015	A0A0A0MY58 and F1SN92	Immunoglobulin heavy constant mu and Salivary lipocalin	28.5 and 25.1	75.1 and 43.5	32.7 and 21.6	28.5
N	6.88 ± 2.44	10.20 ± 1.43	0.009	F1SN92	Salivary lipocalin	54.9	152.5	21.6	26
P	17.51 ± 4.27	3.40 ± 2.10	0.0005	P81245	Odorant-binding protein	75.1	199.5	17.7	18
R	1.22 ± 1.63	4.00 ± 2.47	0.033	A0A4X1TU02	Salivary lipocalin	57.5	143.4	21.6	16.5
T #	14.15 ± 4.91	8.33 ± 4.70	0.043	A0A286ZRW6 and A0A287ASS4	Double-headed protease inhibitor, submandibular gland-like and Prolactin inducible protein	29.4 and 36	58.31 and 56.35	13.3 and 12.4	13

ni—protein failing identification by MS; # in the tryptic mixture, peptides corresponding to more than one protein were observed in the spectra, indicating that more than one protein was present in the band.

From the 1DE analysis, it was evident an increase in salivary lipocalin and IgA bands in *E. coli* diseased pigs, whereas bands containing proteins such as odorant-binding protein, a protease inhibitor from the submandibular origin and/or prolactin inducible protein were present in decreased levels in these animals.

3.3. Two-Dimensional Protein Profile (2-DE)

After gel analysis, it was possible to consider 127 protein spots constantly present in the different pool samples, which were compared between healthy and *E. coli* sample pools. Testing the possibility of separation of the two groups using principal component analysis, it is possible to see that the two components explain 46.98% of data variability (Supplementary Figure S1).

Through the between-subjects test (independent *t*-test), a total of 35 protein spots were observed to present a statistically significant difference ($p < 0.05$) (Figure 2). Among these, 15 protein spots were increased in *E. coli* animals, whereas 20 were decreased. The level of variation, as well as the salivary proteins identified, are presented in Table 2.

Table 2. Protein spots differently expressed between healthy and *E. coli*-diseased pigs.

Spot Number	Fold Change	Group with Higher Level	*p*-Value	Protein (Entry Name)	UNIPROT Protein Accession Number	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
237	4.24	*E. coli*	5.24×10^{-5}	Adenosine deaminase and salivary lipocalin	A0A0B8RW47 and A0A4X1TU02	22.5 and 15.7	39.5 and 23.8	40.9 and 21.6	17.5
33	1.72	Healthy control	0.000222		n.i.				
185	2.30	*E. coli*	0.00063	IgA constant region	A0A287B626	3.8	23.6	44.2	27.5
188	2.41	*E. coli*	0.000733	IgA constant region	A0A287B626	2.6	20.0	44.2	27.5
41	3.29	Healthy control	0.000763	Albumin (whole)	A0A286ZT13	41.1	327.8	68.2	74.5
145	1.56	Healthy control	0.000794	Carbonate dehydratase VI	A0A4X1W7S7	15.1	39.5	34.7	36.0
40	2.72	Healthy control	0.000871	Albumin (whole)	A0A286ZT13	41.1	327.8	68.2	74.5
202	2.28	*E. coli*	0.000887	Ig-like domain-containing protein	A0A287A4Y3	15.4	41.4	24.7	26.0
44	3.03	Healthy control	0.001118	Lactoperoxidase	A0A480RK36	6.6	48.7	80.3	74.5

Table 2. *Cont.*

Spot Number	Fold Change	Group with Higher Level	*p*-Value	Protein (Entry Name)	UNIPROT Protein Accession Number	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
196	2.97	*E. coli*	0.001675	Albumin (fragment) and salivary lipocalin	A0A286ZT13 and A0A4X1TU02	13.6 and 23.5	100.5 and 31.5	68.2 and 21.6	26.0
200	2.34	*E. coli*	0.002233	Albumin (fragment) and salivary lipocalin	A0A286ZT13 and A0A4X1TU02	13.6 and 23.5	100.5 and 31.5	68.2 and 21.6	26.0
45	2.10	Healthy control	0.002679	Lactoperoxidase	A0A480RK36	6.7	48.8	80.3	74.5
43	3.30	Healthy control	0.003706	Lactoperoxidase and polymeric immunoglobulin receptor	A0A480RK36 and A0A0E3M2Q4	7.5 and 6.5	45.5 and 37.3	80.3 and 67.3	74.5
194	1.71	*E. coli*	0.004477	Albumin (fragment)	A0A286ZT13	7.8	64.3	68.2	26.5
31	2.29	Healthy control	0.005324			n.i.			
47	1.66	Healthy control	0.005441	Lactoperoxidase and polymeric immunoglobulin receptor and	A0A0E3M2Q4 and A0A480RK36	12.3 and 4.9	86.1 and 34.7	67.3 and 80.3	74.0
184	1.86	*E. coli*	0.007066			n.i.			
203	2.94	*E. coli*	0.007897	Ig-like domain-containing protein	A0A287A4Y3	18.5	34.0	24.7	26.0
38	2.12	Healthy control	0.009251	Albumin (whole)	A0A286ZT13	41.1	327.8	68.2	101.0
32	1.50	Healthy control	0.012577			n.i.			
37	2.03	Healthy control	0.01381			n.i.			
155	1.51	Healthy control	0.015799	Carbonic anhydrase	A0A4X1W9S1	11.0	27.7	36.3	36.0
179	1.96	Healthy control	0.020918	Carbonate dehydratase VI	A0A4X1W7S7	11.5	47.2	34.7	27.5
73	1.78	Healthy control	0.021757	Alpha-amylase	F1S573	30.1	146.0	55.8	58.0
74	1.39	Healthy control	0.026339	Alpha-amylase	F1S573	30.9	123.4	55.8	58.0
235	2.49	*E. coli*	0.030702	Adenosine deaminase and salivary lipocalin	A0A0B8RW47 and A0A4X1TU02	22.5 and 15.7	39.5 and 23.8	40.86 and 21.61	18.0
130	2.26	*E. coli*	0.033046			n.i.			
146	1.38	Healthy control	0.037883	Carbonate dehydratase VI	A0A4X1W7S7	9.8	25.6	34.7	36.0
77	1.67	Healthy control	0.039092			n.i.			
190	1.75	*E. coli*	0.040238	Albumin (fragment)	A0A286ZT13	9.2	61.0	68.2	26.0
239	2.94	*E. coli*	0.042094	Salivary lipocalin	F1SN92	4.4	24.51	21.6	17.5
170	1.36	*E. coli*	0.046073			n.i.			
72	2.01	Healthy control	0.046326			n.i.			

Note: n.i. means spots that were not identified.

Taking together the 2DE results, it is possible to observe that *E. coli* pools presented higher expression levels of spots identified as lipocalin, adenosine deaminase, IgA, and albumin peptides. On the other hand, spots containing alpha-amylase, carbonic anhydrase, carbonate dehydratase VI, and whole albumin were decreased in pools from the diseased animals.

3.4. Validation

The measurements of salivary ADA activity showed significantly higher activity levels in pigs with diarrhoea caused by *E. coli* (median 2712 U/L, minimum–maximum range 1293–19936 U/L) compared with healthy pigs (median 881.6 U/L, minimum–maximum range 60.8–2435 U/L) ($p < 0.001$) (Figure 3).

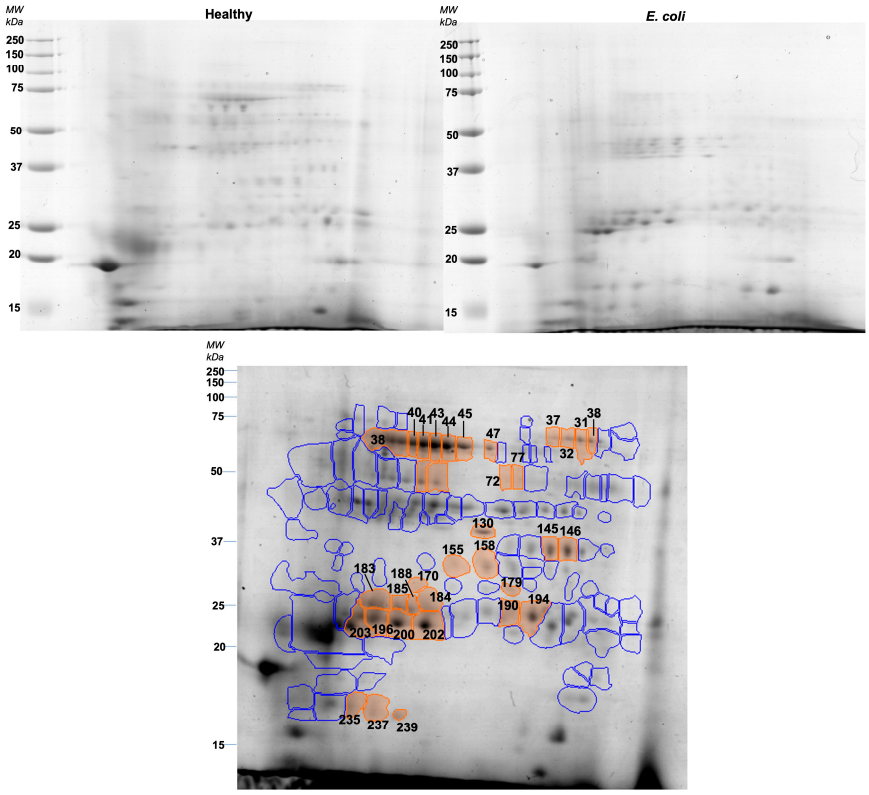

Figure 2. Representative gels of healthy (upper left) and *E. coli* (upper right) pools. The lower image represents the reference gel with protein spots differently expressed between groups (orange) and spots that did not show differences between groups (blue).

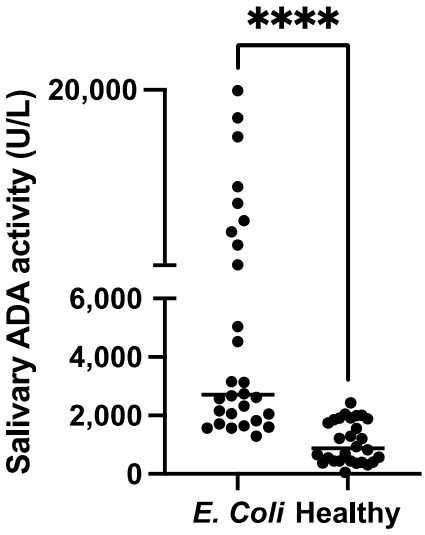

Figure 3. Comparison of the salivary adenosine deaminase activity (ADA) in pigs with diarrhoea caused by *E. coli* and healthy pigs. The plot shows the individual values of each group. **** $p < 0.001$.

4. Discussion

In this report, changes in various proteins in the saliva of pigs with diarrhoea caused by *E. coli* were detected. To the authors' knowledge, this is the first report in which a proteomic analysis of saliva is performed in pigs with diarrhoea due to *E. coli* infection and where changes in salivary proteins in this disease are described. The proteomic approach of this study used 1DE and 2DE gels. 1DE allows the separation of proteins only according to their molecular masses and the entry into the gel of a broad range of proteins, whereas 2DE may

not be able to separate proteins with extreme isoelectric points or higher hydrophobicity. The lower requirement for total protein allowed testing samples at the individual level with this technique. On the other hand, 2DE allows for a more detailed protein profile, obtained after proteins are separated both by their charge and mass. Both 1DE individual samples and 2DE sample pools were run in duplicate to minimize the effect of technical errors inherent to the techniques.

From the 1DE analysis, it was evident that there was an increase in salivary lipocalin and IgA bands in *E. coli*-diseased pigs, whereas bands containing proteins such as odorant-binding protein and/or prolactin-inducible protein were present in decreased concentrations in these animals.

Lipocalin (LCN) family proteins are small proteins (18–40 kDa) expressed in numerous tissues and involved in multiple processes (i.e., inflammation, detoxification, and immune activation) by transporting hydrophobic molecules (e.g., steroids, retinoids, or lipids) to cells [12]. Some members of this family of proteins such as lipocalin-2 (also known as neutrophil gelatinase-associated lipocalin) are considered acute phase proteins showing increases in inflammation [13]. Lipocalin-2 is increased in the serum of humans with inflammatory bowel disease and is correlated with the activity of this disease [14,15]. In addition, it has been described to capture bacterial siderophores produced by pathogenic bacteria, such as *E. coli* and, indeed, Lcn2-deficient mice are prone to infection and sepsis [16]. Although in our study LCN increased, in a previous report it was observed a decrease of LCN in the saliva of pigs with *Streptococcus suis* infection [4]. Further studies should be undertaken to elucidate the mechanisms involved in the change in LCN since in some cases, such as in the *Streptococcus suis* infection, the decrease of lipocalin could indicate a high susceptibility to worsening sepsis [4].

Odorant binding protein (OBP) is involved in olfaction and defence against oxidative injury. In addition, this protein has been related to inflammation, showing a decrease in lungs in bovine after LPS administration. This decrease in OBP levels may be an additional mechanism to allow inflammatory mediators to stimulate neutrophil recruitment and oxidative burst in the lung and possibly in other tissues [17].

Prolactin-inducible protein (PIP) is a small (17 kDa) single polypeptide chain protein expressed in various human body parts, including the salivary gland, lacrimal gland, trachea, prostate, muscle, mammary glands, and lungs [18]. Its expression is upregulated by prolactin and androgens, and oestrogens downregulate it. It is involved in the immune response and can inhibit the growth of bacterial species [19]. The decrease in PIP found in our study could be related to a decrease in prolactin, which has been described in pigs with inflammation [20] and humans with sepsis [21].

In 2DE, lipocalin, adenosine deaminase (ADA), IgA, and albumin peptides were increased in the saliva of pigs with *E. coli*, whereas spots containing carbonic anhydrase, carbonic dehydratase VI, alpha-amylase, and whole albumin were decreased in pools from the diseased animals. ADA was selected to validate the proteomic results due to the existence of an automated assay validated for pigs [22]. ADA increases inflammation and sepsis in the saliva of pigs [4,11]. The increase in ADA found in our proteomic study was also confirmed in the larger population of pigs with diarrhoea with *E. coli* compared to healthy pigs, corroborating the higher levels of this protein in saliva in this disease, possibly reflecting activation of inflammation and the immune system. In addition, IgA, which is produced by the immune system to prevent the invasion of pathogenic microbes and is found in large amounts in the mucosal secretions of the gastrointestinal tract and saliva, was increased in our study. This could agree with other reports that have described an increase in IgA in mucosal secretions after an *E. coli* infection [23].

Carbonic anhydrase (CA; EC 4.2.1.1) represents a group of enzymes that catalyse the reversible hydration/dehydration of CO_2 and water. It is involved in the regulation of colonic electrolyte transport and inhibition of CA activity in the colonic mucosa can lead to a decrease in water absorption [14,15]. In addition, CA has been suggested to mediate the colonic absorptive response to changes in systemic acid-base balance. In this line, human

patients with mild or moderate ulcerative colitis showed a significant reduction of the CA isoenzyme I mRNA and protein and total CA activity in the inflamed mucosa compared to controls [24]. Therefore, it could be postulated that the decreases in CA found in our report would be related to damage in the intestinal mucosa. Carbonic dehydratase VI, which is considered an isoenzyme of CA, was also decreased in our study, possibly due to the reasons described above.

A decrease in spots containing alpha-amylase was also observed in the diseased animals. Usually, the activity of alpha-amylase in the saliva is increased in situations of stress and disease in pigs [25]. The divergence of the decrease found in the amount of amylase in our study compared with the increases in the activity reported in other diseases could be due to the divergences between the amount of one enzyme and its activity, which can occur especially in the case of alpha-amylase [26]. In fact, the 2DE spots represent the relative amount of the forms of the protein, which may not be the ones most contributing to the enzymatic activity. Regarding the albumin, there was a decrease in whole albumin but an increase in peptides with MW lower than the MW of the primary form of albumin. This could indicate that albumin could have some proteolysis in the saliva of diseased pigs. Increases in albumin fragments in the blood due to albumin proteolysis have been described in some diseases such as renal failure [27].

Overall, in our report, we found changes in proteins in saliva related to inflammation and the immune system, as have been described in saliva in pigs with sepsis experimentally induced by LPS administration and other infectious diseases such as *S. suis* infection [3,4].

This report has a limitation in the use of pools for 2D, which does not accurately represent the contribution of the different individual samples. However, there was an agreement in proteins such as lipocalin and IgA between the results of 1D (that was made in individual samples) and 2D gels; also, the increases in ADA in 2D gels were later confirmed by an automated assay in a larger number of individual samples. Further studies involving the validation of a larger number of proteins and a larger number of animals should be made to corroborate the results of our report. In this line, although the study of diseased animals on farms provides a real picture of the disease under field conditions, ideally additional studies in which *E. coli* infection is induced in experimental pigs should be performed to confirm the findings of this report. In addition, it would be of interest to perform additional studies to evaluate possible different proteoforms and protein species to better elucidate the proteome complexity in the saliva of healthy pigs and pigs with diarrhoea caused by *E. coli*.

5. Conclusions

It can be concluded that pigs with diarrhoea caused by *E. coli* infection have changes in proteins in their saliva that can be detected by gel proteomics. These proteins are related to various pathophysiological mechanisms activated in diseases such as inflammation and immune function, and could potentially be biomarkers that could help detect and monitor this disease.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/proteomes11020014/s1, Figure S1: Distribution of sample pools among the two first components obtained by principal component analysis.

Author Contributions: Conceptualization, J.J.C., S.M.-S. and E.L.; methodology, M.R., M.J.L.-M., A.O.-B., J.J.C. and E.L.; software, M.R., M.J.L.-M. and A.M.-P.; validation, M.R., M.J.L.-M., A.O.-B. and E.L.; formal analysis, M.R., M.J.L.-M., A.O.-B. and E.L.; investigation, J.J.C., S.M.-S., A.M.-P. and E.L.; resources, J.J.C. and E.L.; data curation, M.R., M.J.L.-M. and A.M.-P.; writing—original draft preparation, M.R., M.J.L.-M., J.J.C. and E.L.; writing—review and editing, M.R., M.J.L.-M., A.O.-B., J.J.C., S.M.-S., A.M.-P. and E.L.; visualization, J.J.C., S.M.-S. and E.L.; supervision, J.J.C., S.M.-S., A.M.-P. and E.L.; project administration, J.J.C., S.M.-S. and E.L.; funding acquisition, J.J.C., S.M.-S. and E.L. All authors have read and agreed to the published version of the manuscript.

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Objective 3

Validation and measurement of various biomarkers of inflammation, oxidative stress, welfare, or muscle damage, with potential application in septic inflammation and study of their possible changes in sepsis and other conditions.

Article 5 (published):

Salivary D-dimer in pigs: validation of an automated assay and changes after acute stress

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Salivary D-dimer in pigs: Validation of an automated assay and changes after acute stress

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ABSTRACT

D-dimer is a peptide found in serum and is derived from the degradation of blood clots. Even though it has been analysed in human saliva, D-dimer has not been previously evaluated in the saliva of any veterinary species, and its source and role remain unknown. The objectives of this research were firstly, to validate the use of an automated method for the measurement of D-dimer in porcine saliva, and secondly, to evaluate whether D-dimer concentration changes in pig saliva after an acute stress stimulus. For this purpose, a complete analytical validation of a commercially-available immunoturbidimetric assay was carried out. In addition, an experimental acute stress model was induced in 11 pigs based on a technique involving restraint by nose-snare immobilisation for 1 min. Saliva samples were subsequently collected at different times and D-dimer, salivary alpha-amylase (sAA) and cortisol were assessed in order to evaluate changes in its concentrations after the stress induction.

The D-dimer automated assay showed adequate reproducibility and sensitivity, with coefficients of variation below 10% and a limit of quantification of 0.167 μg/mL fibrinogen equivalent units (FEU). It also showed a high accuracy, determined by linearity under dilution and recovery tests. In the stress model, a significant increase ($P < 0.05$) in salivary D-dimer 15 min after the stress stimulus and a positive correlation between D-dimer and sAA ($r = 0.51$; $P < 0.001$) were observed. These results indicate that D-dimer can be measured in porcine saliva with an automated method and suggest that its concentration can be influenced by stressful conditions.

Introduction

Saliva is a widely used sample in animal welfare evaluation due to its ease of collection and its non-invasive nature (Lac, 2001; Palme, 2012). This is especially important in the case of pigs, where blood collection causes a significant stress response due to the necessary animal immobilization required, making saliva collection a better option for stress assessment (Merlot et al., 2011).

D-dimer is a peptide which is considered a biomarker of fibrinolysis because it is derived from the degradation of blood clots. Measurements of this peptide are mainly undertaken in humans and some veterinary species such as dogs and cats for the detection of thrombotic diseases or disorders such as pulmonary thromboembolism and disseminated intravascular coagulation (Griffin et al., 2003; Adam et al., 2009). Its quantification is usually made on blood samples, although one study in humans reported the measurement of this analyte in saliva (Zhang et al., 2013). To the authors' knowledge, D-dimer has never been measured in saliva of any veterinary species. In addition, there are not previous reports of the evaluation of possible changes in salivary D-dimer after stress.

In humans, stressful situations can trigger the clotting system, leading to increases of D-dimer in blood (von Känel et al., 2009; Austin et al., 2013). The release of catecholamines into the blood after certain types of acute stress is reported to increase procoagulant events such as platelet clumping (Austin et al., 2011) and procoagulant elements such as von Willebrand factor and factor VIII (Von Känel and Dimsdale, 2003). These catecholamines can also produce decreases in antithrombin III (von Känel et al., 2002), and together these changes can lead to a hypercoagulable state (von Känel et al., 2019). These findings are consistent with the results of a study in which mice were exposed to 20 h of restraint stress. After the restraint, they displayed an increase in analytes related to the coagulation system that was not found when a previous chemical sympathectomy had been performed (Stämpfli et al., 2014). A state where blood

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coagulation is promoted could play an important role within the 'fight or flight' response after a stressful event, trying to minimize blood loss in case of injury (Cannon and Mendenhall, 1914; Austin et al., 2013).

The aim of this study was to evaluate whether D-dimer could be measured in the saliva of pigs and whether its concentration would change after the application of an acute stress stimulus. For this purpose, a complete validation of an immunoturbidimetric method for the measurement of salivary D-dimer in this species was performed. In addition, an experimental model of acute stress was induced to evaluate possible changes in D-dimer concentrations. In this model, cortisol concentrations and salivary alpha-amylase (sAA) activity were measured to assess the stress responses of the hypothalamic-pituitary-adrenal axis (Palme, 2012) and the autonomic nervous system (Contreras-Aguilar et al., 2018) respectively, and any correlation between D-dimer and these responses.

Materials and methods

Animals and experimental acute stress model

Six males and five female Large White pigs from the University of Murcia in the mid-fattening period were used. Their age and weight data was normally distributed with a mean age (± standard deviation, SD) of 104.8 ± 10.0 days and mean bodyweight (± SD) of 78.3 ± 6.3 kg. Pigs are typically kept from 9 to 24-weeks-old, before being sent for commercial slaughter. No clinical signs of disease were detected in the animals prior to sampling. Pigs had access to a nutritionally balanced diet and *ad libitum* water from nipple drinkers and were housed in concrete slatted-floor pens with a manure pit system. For this study, animals from two different rooms were chosen, where each room had 10 pens containing 14 pigs per pen, with a minimum space of 0.65 m^2 per animal[1] and an average temperature of 23 ± 2 °C. This study was approved by the Ethical Committee of the University of Murcia (Approval number, CEEA 171/2015; Approval date, April 2015).

The stressor stimulus consisted of a temporary restraint of the pigs with a nose-snare, a procedure used in veterinary practice which has proved to cause high levels of stress in pigs (Escribano et al., 2013; Martínez-Miró et al., 2016) manifested by high-pitched vocalisations and increased concentrations of cortisol in plasma and saliva samples (Merlot et al., 2011; Contreras-Aguilar et al., 2018).

Sampling procedures

The animals were chosen by convenience sample. In order to avoid the effect of possible stress due to observation of the nasal-snare procedure in other animals of the same pen, only one pig per pen was selected. Generally, pigs readily chew the sponge without prior training due to their natural curiosity. In this study, the animal in each pen that showed most curiosity (by chewing on the sponge) was the animal used. Saliva samples were collected using Salivette tubes (Sarstedt, Nümbrecht, Germany) with sponges, as reported previously (Escribano et al., 2013).

In all animals, saliva samples were collected before the induction of the stress (TB, baseline time), during the immobilization with the nose-snare (T0), and at 15 min (T15) and 30 min (T30) after the immobilization. All TB samples were collected before starting the procedures of nose-snaring in order to avoid possible influences of vocalisations from other animals due to the nose-snare restraint. At T0, one operator collected the saliva (by introducing the rod with the sponge into the sampled pig's mouth and its margins) while a second operator was applying the nose-snare immobilisation. The restraint of the pigs was performed for 1 min, following the procedure described in previous studies (Fuentes et al., 2011; Escribano et al., 2013). At TB, T15 and T30 each pig was allowed for 1 min to gently chew on a sponge clipped to a flexible thin metal rod of approximately 10 cm of length that was held by an operator located inside the pens. The experimental period lasted 3 h, from 9:00 am to 12:00 pm.

After sample collection, the sponges were introduced in the tubes and were stored in ice until arrival at the processing laboratory, where the tubes were centrifuged at 3000 × *g* for 10 min at 4 °C. Saliva samples were transferred into Eppendorf tubes and stored at −80 °C until the analysis was performed.

[1] See: European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. http://conventions.coe.int/treaty/en/treaties/html/123.htm (Accessed 11 May, 2020)

Salivary D-dimer analysis

D-dimer analysis was performed using a latex enhanced immunoturbidimetric assay (Diazyme Laboratories, California, United States) based on specific anti-D-dimer monoclonal antibodies that bind D-dimer peptides. The degree of turbidity caused by agglutination is measured optically and it is proportional to the amount of D-dimer present in the sample. The method was adapted to an automated analyzer (Olympus Diagnostica GmbH AU600, Beckman Coulter). Results were expressed as fibrinogen equivalent units (FEU) in μg/mL.

Salivary cortisol analysis

Cortisol was measured for comparative purposes and its analytical determination was performed with an immunoassay system (Immulite 1000, Siemens Healthcare Diagnostic) which uses a solid-phase competitive enzyme-amplified chemiluminescent immunoassay. It was previously validated for its use in porcine saliva (Escribano et al., 2012). Results were expressed in μg/dL.

Salivary alpha-amylase analysis

Salivary alpha-amylase activity was also measured for comparative purposes in an automated analyzer (Olympus Diagnostica GmbH AU600, Beckman Coulter, Ennis, Ireland) using a colorimetric commercial kit (Alpha-Amylase, Beckman Coulter) previously validated for pigs (Fuentes et al., 2011), and results were expressed in U/L.

Analytical validation of D-dimer assay

Reproducibility

Intra-assay precision was determined by measuring two pools of porcine saliva samples with a high (3.63 μg/mL FEU) and a low (0.56 μg/mL FEU) concentration of D-dimer respectively, five times each one in a single analytical run, as described by Escribano et al. (2012). The same pools were used to calculate inter-assay precision, measuring them on five different days within a 15-day period. Pools were frozen in aliquots that were thawed as required for each analytical run to avoid any possible variation due to repeated freeze–thaw cycles. The results of intra-assay and inter-assay precision were expressed as the coefficient of variation (CV), calculated as the SD divided by the mean of the values of the different replicates and then multiplied by 100.

Sensitivity

The limit of blank (LoB) was calculated by measuring distilled water ten times in a single analytical run and then calculating the mean plus two standard deviations (SD). The lower limit of quantification (LoQ) was estimated with a serially diluted saliva sample with a high concentration of D-dimer (2.44 μg/mL FEU), measuring each dilution five times in the same analytical run. The LoQ was the lowest concentration that could be measured above the LoB with a CV below 20%, as previously reported (Contreras-Aguilar et al., 2017).

Accuracy

Accuracy was indirectly estimated by linearity under dilution and a recovery study because no reference assay is available for D-dimer in pigs. Linearity under dilution was determined by making serial dilutions (Parra et al., 2005) of a saliva sample and a pool of saliva samples with an intermediate (1.12 μg/mL FEU) and a high (3.69 μg/mL FEU) concentration of D-dimer, respectively. Results were then compared with those expected by linear regression analysis and a coefficient of determination (r^2) was calculated. A recovery experiment was also performed, as previously reported (Parra et al., 2005), using a saliva sample with a high (1.60 μg/mL FEU) and another sample containing a low (0.30 μg/mL FEU) concentration of D-dimer. The high concentration D-dimer sample was diluted twofold (50%), fourfold (25%), and tenfold (10%) with the low concentration D-dimer sample (50%, 75% and 90%, respectively). In addition, the low concentration D-dimer sample was diluted fourfold (25%) with the high concentration D-dimer saliva sample (75%). Afterwards, observed and expected D-dimer concentrations were compared and percentages of recovery were calculated.

Statistical methods

Intra and inter-assay CVs, arithmetic means, SD, LoB and LoQ were calculated in Microsoft Excel. Statistical analyses and the regression analysis were performed using GraphPad Prism version 8 (GraphPad Software).

D-dimer, cortisol and sAA results obtained from the stress model were evaluated for normality of distribution using the Shapiro-Wilk test, and showed a non-parametric distribution. Thus a paired Friedman nonparametric test followed by a Dunn's multiple as a post-test was performed to determine whether the values of the three analytes measured at the different times showed statistically significant differences. Also, a Spearman correlation test with cortisol and sAA was undertaken to determine whether there was a relationship between the response of D-dimer and those of sAA and cortisol. The significance level used in all the analyses was $P < 0.05$.

Table 1
Intra-assay and inter-assay reproducibility obtained in porcine salivary D-dimer measurements in specimens with low and high concentrations of the analyte.

Specimens	Intra-assay		Inter-assay	
	Mean (μg/mL FEU)	CV (%)	Mean (μg/mL FEU)	CV (%)
Low concentration	0.22	7.40	0.26	7.85
High concentration	3.63	1.92	3.48	3.30

FEU, Fibrinogen equivalent units; CV, Coefficient of variation.

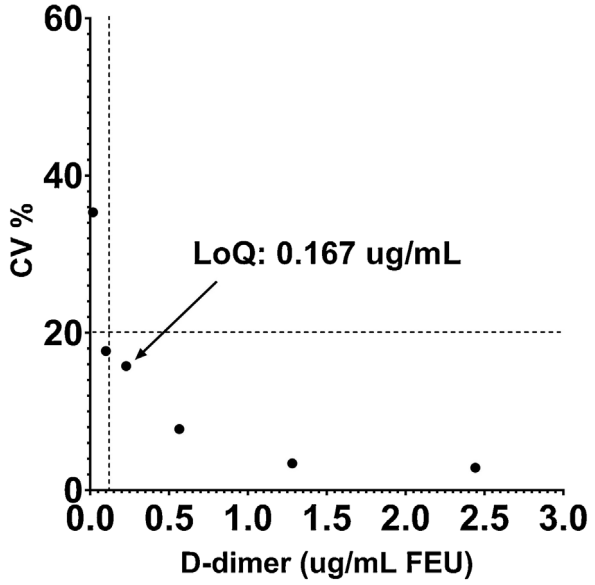

Fig. 1. Dilution series to determine the lower limit of quantification (LoQ) of D-dimer in saliva samples (0.167 μg/mL FEU). Horizontal line shows the highest coefficient of variation (CV) accepted (20%) for the LoQ estimation, while vertical line represents the limit of blank (LoB) (0.129 μg/mL FEU).

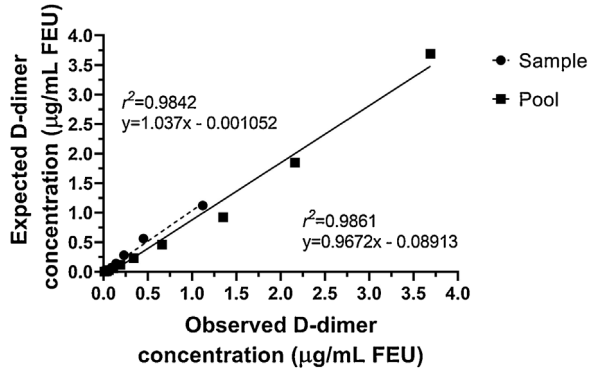

Fig. 2. Linearity under dilution of a saliva sample and a pool of saliva samples, with an intermediate and a high concentration of D-dimer (1.12 μg/mL FEU and 3.69 μg/mL FEU, respectively). The obtained salivary D-dimer concentration is represented on the X-axis, and the expected salivary D-dimer concentrations are represented on the Y-axis. r^2 = coefficient of determination.

Results

Analytical validation of D-dimer assay

Results from the reproducibility study are shown in Table 1. Intra-assay variation showed CVs of 7.40% and 1.92% for the low and high D-dimer concentrations samples respectively, whereas CVs of 7.85% and 3.30% for the low and high D-dimer concentrations samples respectively were found in the inter-assay variation. The LoB of the method was found to be 0.129 μg/mL FEU, and the LoQ 0.167 μg/mL, which was the lowest D-dimer concentration measured with a CV <20%, and above the LoB (Fig. 1). Linear regression equations of observed (measured) salivary D-dimer concentration (X axis) *vs* expected salivary D-dimer concentration (Y axis) are shown in Fig. 2. Coefficients of determination obtained from the sample and the pool were r^2 = 0.9861 and r^2 = 0.9842 respectively. Results of the recovery study are shown in Table 2. Salivary D-dimer concentration decreased proportionally in the diluted sample as the amount of low concentration sample was added. In addition, a range from 95.01% to 106.88% was observed in the recovery test.

Experimental acute stress model responses

Changes in D-dimer, cortisol, and sAA values after the nose-snare immobilization are presented in Figs. 3a–c. After the experimental acute stress model, increases in values of the three analytes were found. There was a significant increase (P = 0.0496) in D-dimer at T15 (0.22, 0.11–0.46 μg/mL FEU; median, interquartile range) when compared to TB (0.08, 0.05−0.11 μg/mL FEU). Cortisol showed a significant increase (P = 0.0496) at T0 (0.936, 0.787−1.22 μg/dL) when compared to TB (0.654, 0.587−0.867 μg/dL). Salivary alpha-amylase (sAA) also showed a significant increase (P = 0.0002) at T0 (817.6, 402.4–1438 U/L) when compared to TB (133.6, 64.80–144.0 U/L). The Spearman correlation test showed a positive correlation between D-dimer and sAA (r = 0.5144; P = 0.0002). No correlation between D-dimer and cortisol (r = 0.274; P = 0.0716) was detected.

The salivary D-dimer concentration increase occurred at different times depending on the animal (Fig. 4). A faster rise was observed in six pigs, with a maximum peak of D-dimer concentration found at T0 or T15, while in three pigs the major increase in D-dimer concentration was found at T30. Two pigs did not show any change in D-dimer concentration.

Discussion

To the authors' knowledge, this is the first report in which D-dimer has been analysed in saliva samples of any animal species. According to the results of our analytical validation, the immunoturbidimetric assay used in this study is able to measure D-dimer in porcine saliva with adequate reproducibility, accuracy and sensibility. Intra and inter-assay CVs were below 10%, within the recommended range for method validation.[2] The observed LoB and LoQ allowed the measurement of D-dimer in the concentration range found in all the saliva samples used in our study. The linearity and high coefficients of determination in serially diluted saliva samples and the results found in the recovery experiment, all within the recommended range (80–120 %)[3], indicated a high accuracy. The only previous study in which D-dimer was measured in saliva samples was performed in humans with an AlphaLISA immunoassay (Zhang et al., 2013), and it showed similar results of reproducibility and accuracy to those obtained with our automated method. The assay tested in this study has the advantage of being automated, allowing a higher precision and sample throughput.

[2] See: US Department of Health and Human Services, Food and Drug Administration (FDA) Guidance for Industry: Bioanalytical method validation, 2001. https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf (Accessed 11 May, 2020).

[3] See: Center for Drug Evaluation and Research (CDER), Guidance Reviewer: Validation of chromatographic methods, 1994. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/reviewer-guidance-validation-chromatographic-methods (Accessed 11 May, 2020).

Table 2
Results of the recovery test performed mixing saliva pools with high and low D-dimer concentrations to evaluate the accuracy of the assay.

% High pool[a]	% Low pool[b]	Observed (μg/mL)	Expected (μg/mL)	Recovery (%)
100	–	1.60	–	–
75	25	1.36	1.27	106.88
50	50	1.00	0.95	105.82
25	75	0.59	0.62	95.55
10	90	0.40	0.42	95.01
–	100	0.29	–	–

[a] % High pool, Saliva pool with a high concentration of D-dimer.
[b] % Low pool, Saliva pool with a low concentration of D-dimer.

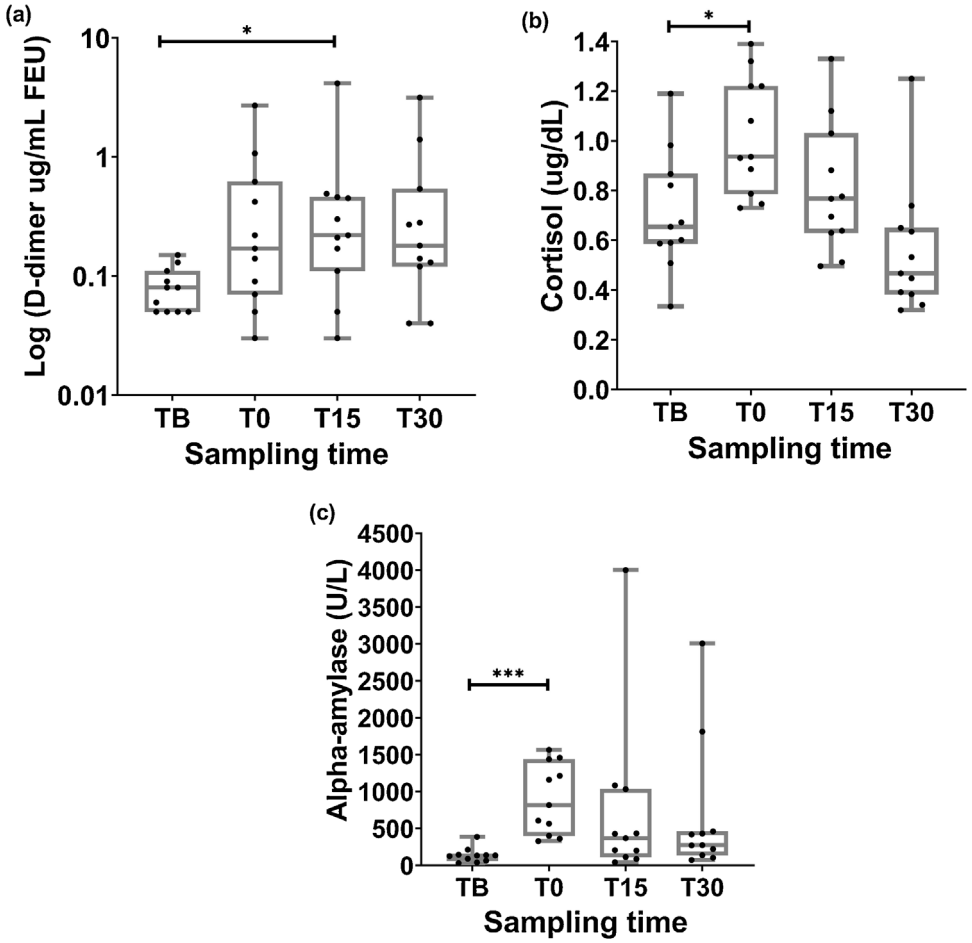

Fig. 3. D-dimer (a), cortisol (b) and salivary alpha-amylase (c) values in saliva obtained from the model of experimental acute stress. TB, basal time; T0, during the application of the stressor; T15 and T30, 15 and 30 min after the application of the stressor. Graphs show medians (line within box), 25th and 75th. percentiles (boxes), min and max values (whiskers) and individual values (points). Asterisks indicate statistically significant differences (*$P < 0.05$). In Fig. 3a, the Y-axis (D-dimer μg/mL FEU) is shown on a logarithmic scale.

D-dimer concentrations generally increased in saliva after the experimental acute stress model tested in our study. The positive correlation of D-dimer concentrations with sAA activity and the lack of correlation with cortisol suggest that this increase could be associated with the autonomic nervous system response to stress, rather than the hypothalamic-pituitary-adrenal axis response. These results would agree with previous studies performed in humans and mice that found that the activation of blood clotting after a stressful event was mainly triggered by an autonomic nervous system response (Stämpfli et al., 2014; von Känel et al., 2019).

With regard to the D-dimer increase among the individual pigs, different kinetics were found in our study, as its peak concentration was observed at different times (T0, T15 or T30) depending on the pig. Similarly, von Känel et al. (2009) found in their acute stress experiment that the increase in D-dimer in human blood was produced at different times in each person. These authors observed that the most delayed increases of D-dimer (approximately 90 min after the stress stimulus) corresponded to individuals with a previous chronic stress status. They hypothesized that a long-lasting stressor could impair fibrinolytic activity producing a slower degradation of blood clots, which would result in a delayed release of D-dimer (von Känel, 2015). Further studies would be of interest to evaluate whether previous exposure of pigs to chronic stress can influence the response of D-dimer after an acute stressful event.

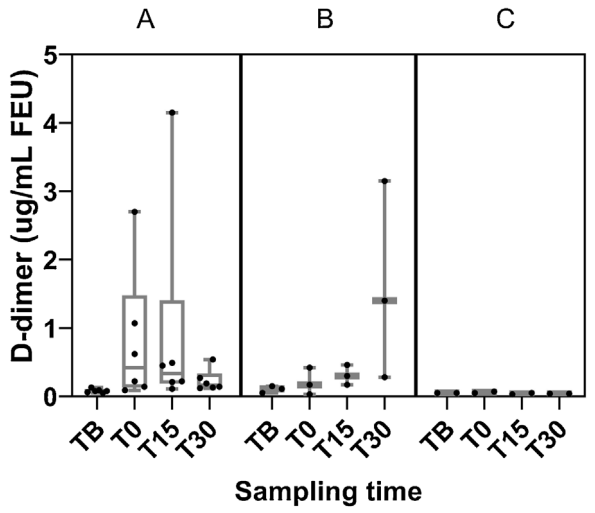

Fig. 4. Salivary D-dimer concentrations found at different times among individual pigs. A rapid increase was observed in 6/11 pigs with a peak at T0 or T15 (A), while 3/11 pigs had the D-dimer peak at T30 (B) and two pigs had no increase in D-dimer (C). TB, basal time; T0, during the application of the stressor; T15 and T30, 15 and 30 min after the application of the stressor. Graphs show medians (line within box), 25th and 75th. percentiles (boxes), min and max values (whiskers) and individual values (points).

Some limitations should be taken into account in this research regarding the small number of animals used in the experimental acute stress model and the lack of paired blood samples, which would have been of interest to evaluate the possible correlation between D-dimer in saliva and in blood. Also, we cannot rule out that the lack of prior training of the pigs in the procedure to collect the saliva could produce some stress in the animals, although external signs of stress were not observed at the first collection (TB). In addition, more studies to test whether the results found in this study may apply to other types of stress from low to moderate levels would be also of merit. Overall, further research should be perform to make a more complete evaluation of salivary D-dimer changes in response to stressful events as well as to clarify the causes and possible implications of higher D-dimer levels in saliva during stress.

Conclusions

This study showed that D-dimer can be measured in saliva samples of pigs with adequate reproducibility, accuracy and sensitivity using an automated immunoturbidimetric assay.

Significant increases in salivary D-dimer were observed after the induction of experimental acute stress with a nose-snare restraint in pigs, indicating this analyte may be influenced by stress. Also, a positive correlation between D-dimer concentrations and sAA activity was found, which suggests that the autonomic nervous system response to stress could be related to the salivary D-dimer increase.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Changes in biomarkers of redox status in saliva of pigs after an experimental sepsis induction

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Changes in Biomarkers of Redox Status in Saliva of Pigs after an Experimental Sepsis Induction

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Abstract: Saliva from pigs is gaining attention as an easy sample to obtain, being a source of biomarkers that can provide information on animal health and welfare. This study aimed to evaluate the changes that can occur in salivary biomarkers of the redox status of pigs with an experimentally induced sepsis. For that, the cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of saliva (FRAS), Trolox equivalent antioxidant capacity (TEAC), advanced oxidation protein products (AOPP), ferrous oxidation-xylenol orange (FOX), peroxide activity (POX-Act), and reactive oxygen-derived compounds (d-ROMs) were measured in the saliva of pigs with experimentally induced sepsis by endotoxin lipopolysaccharide (LPS), non-septic inflammation induced by turpentine, and in healthy individuals before and after 3 h, 6 h, 24 h, and 48 h. AOPP, POX-Act, and d-ROMs in the sepsis group were higher than in the control from 3 h to 24 h after the inoculation. CUPRAC, FRAS, and TEAC were higher in sepsis than the control group at 24 h. These changes were of higher magnitude than those that occurred in the turpentine group. In conclusion, our findings reveal that sepsis produces changes in salivary biomarkers of redox status, which opens the possibility of using them as potential biomarkers in this species.

Keywords: antioxidants; cupric; ferric; inflammation; oxidative stress; peroxides

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1. Introduction

The use of saliva for the determination of biomarkers has gained special attention in the last decade due to its easy collection, being of particularly high interest in pigs, in which blood collection is highly stressful. In addition, it has been shown that the saliva matrix is appropriate for health monitoring and disease diagnosis [1,2].

Infectious diseases in pigs are possibly the most important health concern in swine production [3,4]. Depending on factors, such as the immune status of the pig, pathogen involved, and environment, the disease can progress to sepsis and death. In addition, the intensification and globalization of the swine industry are being accompanied by a global spread of pathogens at local and international scales, which, together with the need for appropriate use of antibiotics and the withdrawal in the European Union of zinc oxide supplementation, highlights the importance of the correct detection and control of sepsis in pigs [5,6].

The endotoxin lipopolysaccharide (LPS) challenge is a validated model of sepsis in pigs [7]. In addition, porcine models of LPS-induced sepsis have been developed to simulate human sepsis [8,9]. The LPS is an outer-membrane component of Gram-negative

bacteria that provokes a systemic inflammatory response, which includes the activation of free-radical-generating molecules in various types of cells that initiate tissue damage [10,11].

Free-radical molecules and other oxidants are produced constantly as a result of the respiratory chain, and the antioxidant system works continually to maintain the oxidant levels under control and without damage to the cells [12]. The imbalance between both the oxidant and antioxidant system is involved in the etiology of various diseases. Several studies have already shown that biomarkers of oxidative status can be measured in the saliva of pigs [13] and other species, such as bovine [14], canine [15], ovine [16], as well as humans [17]. To investigate the effects of a natural antioxidant compound during human sepsis, the LPS-induced model in piglets was used and various redox biomarkers were evaluated in serum [18]. However, to the author's knowledge, no information is available yet about the changes that occur in the oxidative status of the saliva of pigs during induced sepsis.

The objective of this study was to evaluate the changes that can occur in biomarkers of redox status in the saliva of pigs with sepsis. For this purpose, an experimental trial using LPS was made. In addition, an experimentally induced non-septic inflammation by turpentine administration was performed in order to compare sepsis with a non-septic inflammatory condition. In these trials, a profile, including four antioxidant (cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of saliva (FRAS), Trolox equivalent antioxidant capacity (TEAC) and uric acid) and four oxidant biomarkers (advanced oxidation protein products (AOPP), ferrous oxidation-xylenol orange (FOX), peroxide activity (POX-Act), and reactive-oxygen-derived compounds (d-ROMs)) was measured.

2. Materials and Methods

2.1. Animals, Housing, and Experimental Design

The experimental protocol was approved by the Ethical Committee on Animal Experimentation (CEEA) of the University of Murcia (CEEA 563/2019) according to the European Council Directives considering the protection of animals used for experimental purposes. In addition, this study complies with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for the care and use of animals.

In total, 15 growing male pigs were included in this study. They were in the mid-fattening period and belonged to the Experimental Farm of the University of Murcia (Murcia, Spain). All of them had water and a balanced diet ad libitum and were housed with a minimum space of 0.65 m^2 per animal (Council Directive 2001/88/CE of 23 October 2001 amending Directive 91/630/CEE concerning minimum standards for the protection of pigs) and a standard temperature of 24 $\pm$ 2 °C. During the study, the pigs were 14 weeks old and had a median weight of 51.5 kg (interquartile range 48–53 kg).

Before starting the experiment, the animals were adapted to the experimental conditions for a week and then randomized and divided into three groups. The first group (n = 5; control group) received saline treatment (2 mL) by intramuscular route. The second group (n = 5; LPS group) received a single dose of 30 ug/kg LPS from Escherichia Coli (LPS; O55:B5, Sigma-Aldrich) reconstituted in sterile saline solution by intramuscular injection [19,20]. In the third group (n = 5, TURP group), 8 mL of TURP (oil of turpentine purified, Sigma–Aldrich) was given by two 4 mL subcutaneous injections in each front flank per animal. All injections were completed between 8 and 9 am. In some of these saliva samples, procalcitonin was measured for a previous study [21].

2.2. Sampling Procedure

Saliva samples were obtained 24 h before (baseline) the saline, LPS, or TURP injections and at 3, 6, 24, and 48 h after. Saliva was collected using a polypropylene sponge clipped to a metal rod. The pigs were allowed to chew the sponge without forcing them. Then the sponges were placed in Salivette tubes (Sarstedt, Aktiengesellschaft & Co. D-51588 Nümbrecht, Germany) and centrifuged at 3000$\times$ g for 10 min. The saliva samples were collected and stored in Eppendorf tubes at −80 °C until analysis.

2.3. Assessment of Salivary Biomarkers of Oxidative Status

The salivary concentration of CUPRAC, FRAS, TEAC, uric acid, and AOPP was determined using automated techniques previously described and validated for the saliva of pigs [13].

The measurement of FOX was based on the oxidation of ferrous to ferric ions by lipid hydroperoxides in the sample as previously published [22] and results were expressed in μmol of peroxide hydrogen per L of the sample (μmol/L).

The POX-Act determination was based on the assay described by Tatzber et al. [23] in which the oxidation of 3,5,3′5′-Tetramethylbenzidine (TMB) by peroxides in the sample is monitored. The results were also expressed in μmol of peroxide hydrogen per L of the sample (μmol/L).

Salivary d-ROMs levels were determined based on monitoring the N,N-Diethyl-p-phenylenediamine radical cation concentration as previously described [24] with results expressed in Carratelli units (Carr units).

The assays were performed using the Olympus AU400 (AU400 Automatic Chemistry Analyser, Olympus Europe GmbH, Hamburg, Germany) and showed lower than 15% imprecision. More details about the assays are described in Table S1.

2.4. Statistical Analysis

Data (Spreadsheet S1) were analyzed using GraphPad Prism software Inc. (GraphPad Prism, version 8 for Windows, Graph Pad Software Inc., San Diego, CA, USA). The normality of the distribution of the results was assessed by using the D'Agostino and Pearson test, giving a nonparametric distribution; therefore, all data were normalized by logarithmic transformation (Y = Log[Y]) before analysis. A two-way analysis of variance (ANOVA) mixed model of repeated measures was performed, and time and treatment were used as random factors to account for multiple observations. Sidak's multiple comparison test was used to compare the groups over time. Tukey's multiple comparison test was applied to the data considering the sampling time as a repeated measure, with fixed effects of treatment. The effects were considered as significant if p-values were <0.05.

3. Results

3.1. Antioxidant Biomarkers

LPS-treated pigs had significant increased concentrations of CUPRAC at 24 h when compared to basal time (p = 0.039, Figure 1a). TEAC showed a tendency to increase (p = 0.056, Figure 1b) at 24 h in comparison to basal time in this group. When compared to healthy controls, the LPS group showed significantly higher concentrations of CUPRAC (p = 0.004; Figure 1a), FRAS (p = 0.028; Figure 1b), and TEAC (p = 0.001; Figure 1c) 24 h after injection.

TURP-treated pigs showed no changes in antioxidant biomarkers throughout the study, except for uric acid (Figure 1d), which was decreased at 48 h in relation to baseline (p = 0.044). There was no significant difference comparing control and TURP-treated pigs at any time point (p > 0.05) (Figure 1).

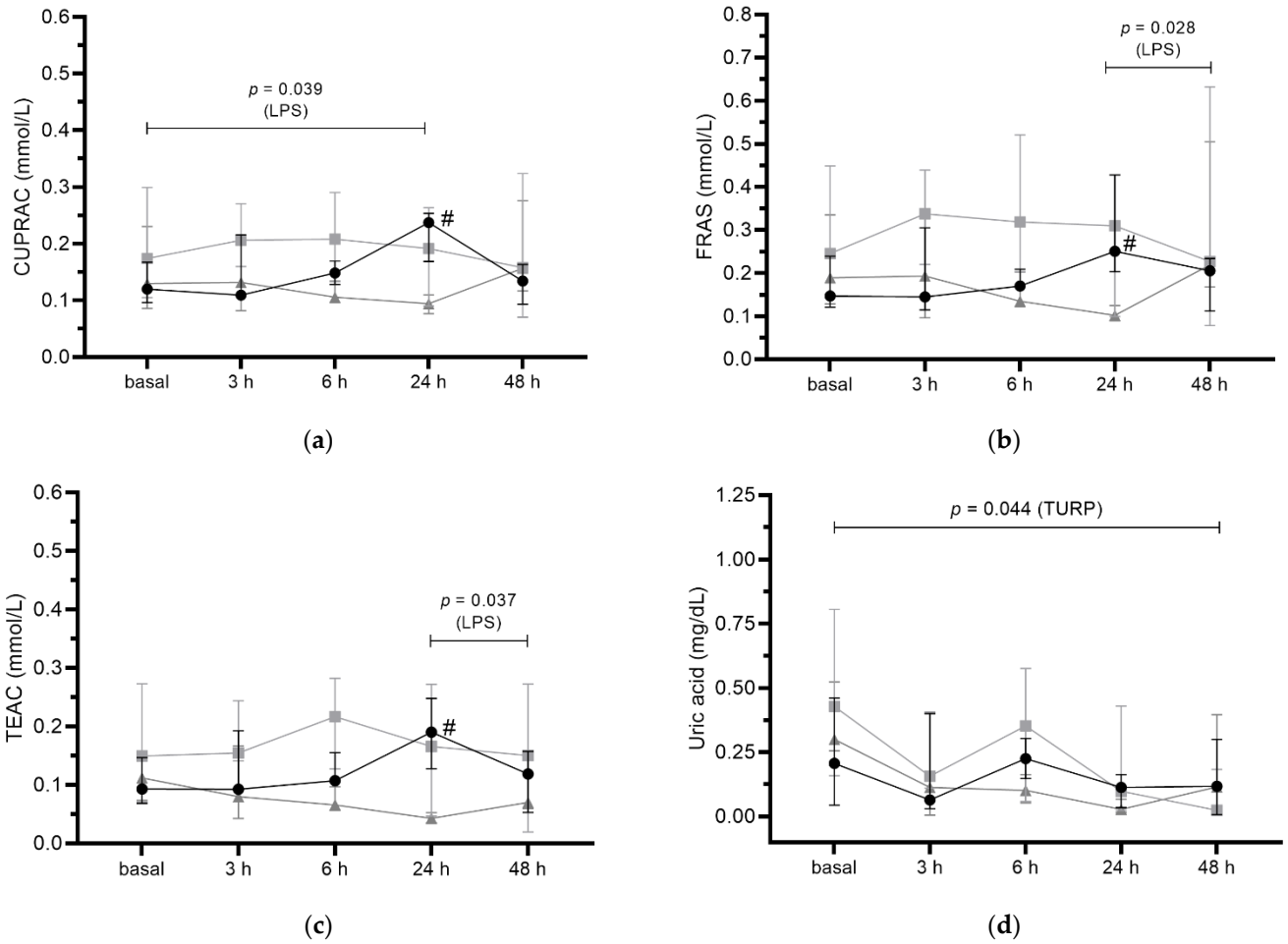

Figure 1. (**a**) Cupric reducing antioxidant capacity (CUPRAC), (**b**) ferric reducing ability of saliva (FRAS), (**c**) Trolox equivalent antioxidant capacity (TEAC), and (**d**) uric acid concentrations in control (▲), lipopolysaccharide (LPS)-treated pigs (●), and turpentine (TURP)-treated pigs (■) before (basal) and after 3 h, 6 h, 24 h, and 48 h the inoculations. The results are presented as median with an interquartile range. **#**, significantly different from the control group ($p < 0.05$; one-way ANOVA with Sidak's multiple comparisons test). Differences between times are indicated by bars and the obtained *p*-value (one-way ANOVA with Tukey's multiple comparison test).

3.2. Oxidant Biomarkers

LPS-treated pigs presented significantly higher POX-Act (Figure 2c) and d-ROMs (Figure 2d) than control at 3 h ($p < 0.039$), 6 h ($p < 0.033$) and 24 h ($p < 0.001$), reaching the highest values at 6 h. They also showed higher POX-Act than TURP-treated pigs at 3 h ($p < 0.036$). When compared to control, the LPS group showed significantly higher concentrations of AOPP ($p = 0.004$, Figure 2a) 24 h after the injections.

There was no significant difference in FOX concentrations between groups and between time points ($p > 0.05$) (Figure 2b).

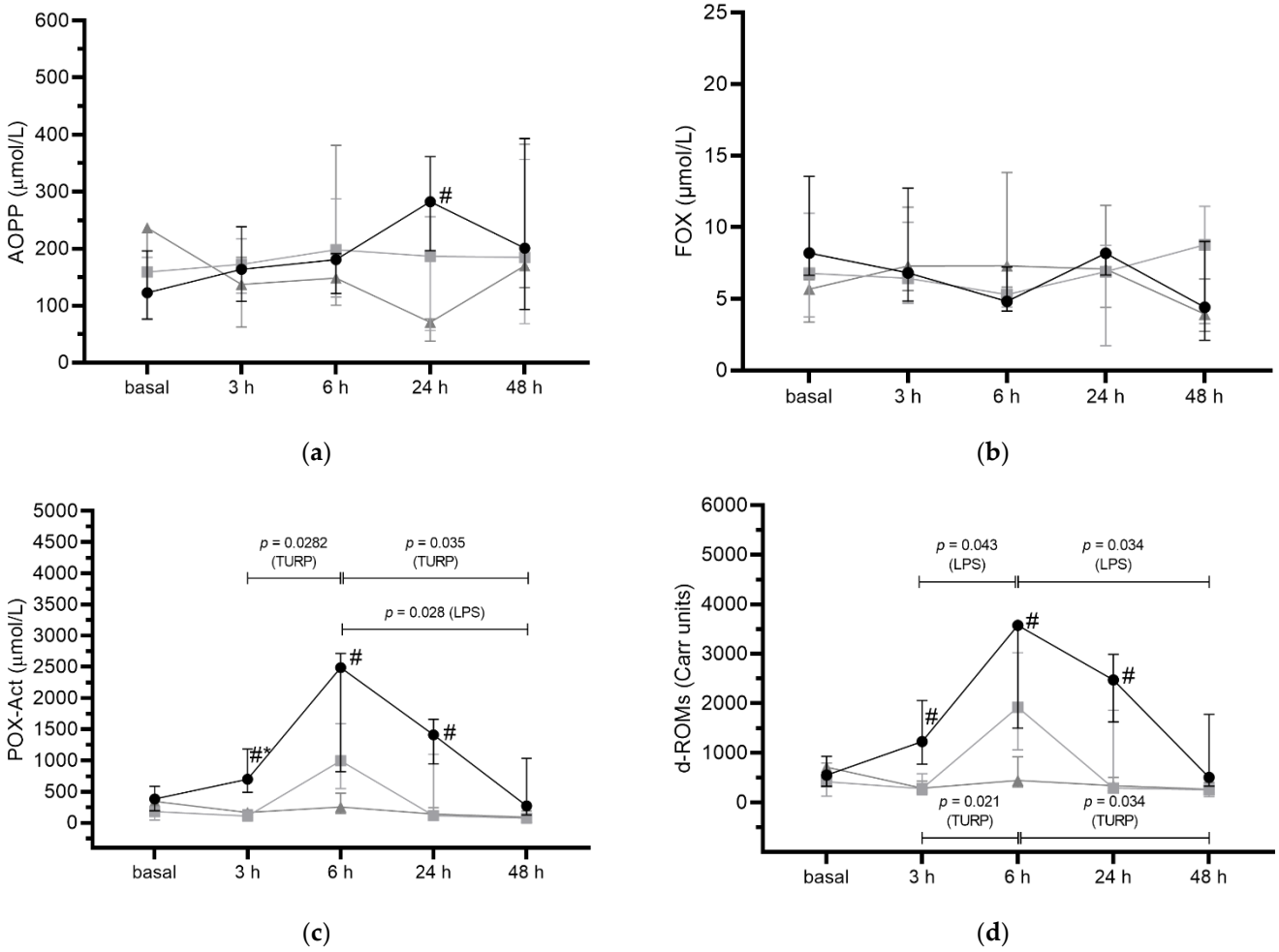

Figure 2. Salivary (**a**) advanced oxidation protein products (AOPP), (**b**) ferrous oxidation-xylenol orange (FOX), (**c**) peroxide activity (POX-Act), and (**d**) reactive-oxygen-derived compounds (d-ROMs) concentrations in control (▲), lipopolysaccharide (LPS)-treated pigs (●), and turpentine (TURP)-treated pigs (■) before (basal) and after 3 h, 6 h, 24 h, and 48 h the inoculations. The results are presented as median with an interquartile range. #, significantly different from the control group; *, significantly different from TURP-treated pigs ($p < 0.05$; one-way ANOVA with Sidak's multiple comparisons test). Differences between times are indicated by bars and the obtained *p*-value (one-way ANOVA with Tukey's multiple comparison test).

4. Discussion

To the best of our knowledge, this is the first study that describes changes in biomarkers of redox status in pigs with sepsis. The pigs in our study showed, after LPS administration, increased body temperature as well as increased white blood cell count and C-reactive protein (Table S2), and the presence of symptoms, such as depression, lethargy, and increased respiratory rate; which proved the validity of LPS administration as a model for sepsis in pigs, as has been previously described [21,25].

The pigs with sepsis showed higher values in three of the oxidants measured in our study (AOPP, POX-Act, and d-ROMs) compared with the healthy control pigs. POX-Act and d-ROMs presented earlier increases, being significantly higher at 3 h after the induction of sepsis, whereas AOPP showed significant increases at 24 h. Although no studies about salivary oxidative biomarkers in pigs with sepsis have been previously reported, pigs with sepsis showed increased 8-Iso-prostaglandin F2α, a lipid peroxidation product, in plasma [26,27]. Furthermore, in piglets with sepsis induced by LPS, liver and lung content of two oxidants, such as malondialdehyde and protein carbonyl, were increased compared

to the control animals [18]. In addition, an increment in oxidants in serum was also observed in human sepsis [28,29]. Therefore, it can be postulated that sepsis induces an increase in oxidants in the organism that can be reflected in saliva.

The increases in oxidant biomarkers in sepsis could be due to the activation of the phagocytic NADPH oxidase complex, leading to the production of reactive nitrogen and oxygen species (RNS and ROS, respectively), and their sustained and excessive production cause damage to endothelial cells and organ failure, which is associated with increased morbidity and mortality in sepsis patients [30]. In our study, the concentrations of oxidants at 48 h in the septic group were similar to the basal and healthy group values, which indicates that production of ROS was not sustained, and that could be associated with the recovery experimented by the pigs in our conditions.

In our report, pigs with induced sepsis showed higher concentrations of CUPRAC, FRAS, and TEAC 24 h after LPS administration compared to the healthy ones. This is in line with previous results in humans, in which septic neonates presented higher levels of antioxidant enzymes in serum, such as superoxide dismutase and glutathione peroxidase [31]. In sepsis, different antioxidant responses can be obtained depending on the severity and outcome [29]. Patients who developed organ dysfunction and did not overcome sepsis showed decreased antioxidants, while those who survived presented increases over time, in line with our findings [32,33]. Therefore, it would be of interest to explore the potential of these biomarkers for predicting the outcome of sepsis.

The increased antioxidant response might be an attempt to counteract the damage that could be caused by the overproduced oxidants observed in this group. Namely, α-tocopherol and ascorbic acid are the first lines of defense against intravascular oxidants involved in sepsis [34]; this could explain our results, since CUPRAC, TEAC and FRAS measure both antioxidants.

In general, our findings reveal an increase in oxidant and antioxidant markers during sepsis in pigs, indicating that salivary biomarkers of redox status change in pigs with this condition. On the other hand, the animals that had local inflammation did not show the differences in redox biomarkers that occurred in the sepsis group. This will indicate that in our experimental conditions, the changes in redox biomarkers produced in sepsis are of a higher magnitude than those of non-septic inflammatory conditions. Further studies should be undertaken in saliva to elucidate if biomarkers of redox status could help to differentiate between septic and non-septic inflammation.

The small number of animals included in this study should be mentioned as a limitation; therefore, this should be considered a pilot study and the results need to be confirmed in a larger population. In addition, it would have been interesting to evaluate the possible correlation of the biomarkers concentrations between saliva and serum, although previous reports in pigs did not find a major correlation in these these analytes between both fluids [35]. Furthermore, the results obtained in this study were not corrected by protein concentration, in line with other studies performed with other biomarkers of oxidative stress in which this procedure was not recommended [36]. However, further research needs to be performed in the future to identify the necessity or not of the correction by protein when biomarkers of oxidative status are measured in animals' saliva.

5. Conclusions

The saliva of pigs during experimental sepsis showed higher levels of the oxidant biomarkers AOPP, POX-Act, and d-ROMs compared to healthy animals. In addition, higher levels of the antioxidant markers CUPRAC, TEAC, and FRAS were also observed in pigs with sepsis. These findings could reflect that there is an increase in oxidants during sepsis in pigs and the mobilization of some antioxidants in order to protect against damage. Overall, this study shows that sepsis can induce changes in pigs' saliva and opens the possibility of using the salivary biomarkers of redox status as potential biomarkers of sepsis in this species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11071380/s1, Table S1: Description of the basis and reagents of each assay performed in the study; Table S2: Rectal temperature, white blood cell count (WBC), and C-reactive protein (CRP) of each animal included in the study before and 3 and 24 h after injection of lipopolysaccharide (LPS), turpentine (TURP), and saline (control group). Data of LPS and TURP groups were previously described [25]; Spreadsheet S1: Raw data.

Author Contributions: Conceptualization, C.P.R. and J.J.C.; methodology, M.J.L.-M., D.E., A.O.-B. and L.F.-M.; validation, C.P.R. and L.G.G.-A.; formal analysis, C.P.R. and L.G.G.-A.; resources, J.J.C.; writing—original draft preparation, M.J.L.-M., C.P.R. and J.J.C.; writing—review and editing, C.P.R., M.J.L.-M., D.E., A.O.-B., L.F.-M. and L.G.G.-A.; visualization, C.P.R. and J.J.C.; supervision, C.P.R.; funding acquisition, J.J.C. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data is contained within this article and supplementary files.

Conflicts of Interest: The authors declare no conflict of interest.

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Novel saliva biomarkers for stress and infection in pigs: Changes in oxytocin and procalcitonin in pigs with tail-biting lesions

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ABSTRACT

There is a need for feasible and reliable measures to improve and evaluate production animal health and welfare. Oxytocin is a promising novel stress-related biomarker and procalcitonin may be a measure of sepsis. Both have potential for use in pigs and can be measured from saliva, which allows on-farm sampling with minimal impact on the animals. The current study sought to further validate these measures using a spontaneous situation that causes both stress and an increased risk for infections in pigs, namely a tail-biting outbreak. Grower pigs on a commercial farm belonging to three different phenotype groups were selected: control pigs from control pens (CC, $N = 30$), control pigs (CTB, $N = 10$), and pigs with tail lesions from pens with a tail-biting outbreak (LTB, $N = 27$). A single sample of saliva was collected from each pig and analysed for a range of biomarkers related to stress, infection, inflammation, and immune activation. Oxytocin tended to be higher in CC pigs than in LTB pigs, while cortisol was higher in CTB than CC pigs. Procalcitonin tended to be higher, and haptoglobin was higher in LTB than in CC pigs. Adenosine-deaminase levels were similar between phenotypes. These results provide further evidence for the link between stress and tail biting, and indicate that tail-biting lesions are potential routes for systemic spread of bacteria. Further research into saliva oxytocin as a stress biomarker and saliva procalcitonin as a sepsis biomarker in pigs is warranted.

1. Introduction

There is a need to develop feasible, reliable, and valid measures of animal health and welfare to improve on-farm diagnostics and to assess the welfare of domestic animals (Guevara et al., 2022). Saliva biomarkers are promising alternatives to more intensive methods, such as blood sampling, as they can be collected with minimal stress to the animal (Cerón et al., 2022).

Cortisol is one of the most commonly used biomarkers for both acute and chronic stress (Merlot et al., 2011; Bahnsen et al., 2021). Cortisol can reliably be analysed from saliva (Escribano et al., 2012). However, not only stressors such as transport (López-Arjona et al., 2020), but also physical exercise can increase cortisol secretion (Allgrove et al., 2008). Further, cortisol secretion can be influenced by the sampling procedure (Ruis et al., 1997), and individual variation and the diurnal secretion pattern of cortisol makes baseline sampling especially challenging (Merlot et al., 2011).

One interesting novel biomarker is oxytocin, a neuropeptide with an important role in reproduction, lactation, and maternal behaviour (Lee et al., 2009). Oxytocin is also linked to social affiliation and has anxiolytic, antinociceptive, and stress-buffering effects (Tops et al., 2007; Tops et al., 2012). Oxytocin is centrally released in response to stress and reduces activity of the hypothalamic-pituitary-adrenal (HPA) axis (Lee et al., 2009). Cortisol and oxytocin are thus interrelated in some contexts; oxytocin increases and provides negative feedback due to increased HPA activation (Quintana and Guastella, 2020; Alley et al., 2019). In domestic animals, oxytocin has primarily been studied during positive experiences, especially social ones (Rault et al., 2017). Recently, López-Arjona et al. (2020, 2021) developed two novel oxytocin assays using a monoclonal and a polyclonal antibody and revealed decreased post-stress oxytocin levels in pigs.

Procalcitonin (PCT) is a widely used biomarker of sepsis in human medicine. PCT is a potential tool for non-invasive detection of sepsis in pigs and has recently been validated for use in pig saliva (López-

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Martínez et al., 2022). PCT levels were increased in both a lipopolysaccharide-induced pig model of sepsis and in pigs with meningitis.

Tail biting in pigs is a problematic abnormal behaviour which can be induced by stressful conditions, particularly in intensive housing systems (Edwards and Valros, 2021). Tail biting and the resulting lesions are connected to increased stress and pain in pigs (Munsterhjelm et al., 2013; Sandercock et al., 2019) and can cause local, secondary, and systemic infections (Boyle et al., 2021). Tail-biting lesions are linked to higher levels of acute phase proteins (APPs) (Heinonen et al., 2010; Carroll et al., 2018) and to higher skin temperature in the tail region (Teixeira et al., 2020), indicating that the lesions cause inflammatory responses.

While it is challenging to separate the underlying stress causing tail biting and the stress caused by tail biting per se, it can be assumed that individuals in a pen with an ongoing tail-biting outbreak suffer from a higher total stress level than in pens with no tail biting. Therefore, our aim was to use spontaneously occurring tail biting as a real-life model to assess oxytocin as a biomarker of chronic stress in pigs. In addition, this model was used to test PCT in saliva as a potential measure of sepsis in pigs. These analytes were compared with more traditional biomarkers related to stress, inflammation, and immune activation, namely cortisol, haptoglobin (Hp), and adenosine-deaminase (ADA) and its isoenzymes. The two main hypotheses were that animals in pens with ongoing tail biting would have lower baseline oxytocin levels but higher cortisol levels than animals from control pens, and that pigs with tail lesions are prone to infections and have increased PCT, Hp, and ADA levels.

2. Material and methods

The study protocol was considered ethically acceptable by the University of Helsinki Viikki Campus Research Ethics Committee (Statement 2/2022).

2.1. Animals and housing

The study was conducted in the growing unit of a commercial piglet-producing farm in Southwest Finland. All pigs were born in similar conditions in standard farrowing crates with partly slatted flooring. Pigs were moved to the growing unit at an age of approximately 27 days. Male pigs were castrated, and pigs were not docked.

Pigs were housed in two-climate pens in groups of about 27 pigs per pen. Feeding was ad libitum with liquid feed from sensor troughs (trough length 2.5 m per pen). Water was available from one cup drinker per pen. Pens were 11.4 m^2 in total, of which 7.6 m^2 was solid concrete floor and the remainder slatted. There was a roofed resting area of 3 m^2 at the back end of the pens. A thin layer of peat bedding covered part of the solid floor area. Peat and a small amount of hay or straw was added to the pens one to two times a day, but hay or straw was barely visible in the pens during sampling. Further, each pen contained a hanging chain with four plastic chewing objects attached to it.

Tail health was assessed at the pen level; pens with >10% of pigs in the pen with a fresh tail lesion >0.5 cm were counted. This situation was true for 7.5 pens per section (median; min = 3, max = 15 of 16 pens per section). At least one shortened, healed tail was present in 0.5 pens per section (median; min = 0, max = 3).

2.2. Selection criteria

Animals were housed in a total of six different rooms. From each room, one to three pens with signs of fresh tail biting were chosen (TB) along with a similar number of control pens with no or very mild signs of tail lesions (C). However, in all rooms it was not possible to find enough C pens to match exactly the number of TB pens. In total, 10C pens from five rooms and 13 TB pens from six rooms were included. From each C pen, three pigs were convenience sampled; these were the first pigs to approach the sampler voluntarily or to allow the sampler to approach without withdrawing. Pigs in the TB pens were additionally separated into three different phenotype groups (Table 1). Whenever suitable pigs could be identified, one, or in some cases two pigs were sampled per phenotype per pen. Only pigs that otherwise appeared clinically healthy were included.

Table 1
Selection criteria and pigs included in the study.

Phenotype	Definition	Number of pigs (F:M ratio)[a]	Number of pens pigs originated from	Number of rooms pigs originated from
Control in control pen (CC)	Pig with clinically intact tail from control pens with no or very mild tail lesions.	30 (18:12)	10	5
Control in tail-biting pen (CTB)	Pig with clinically intact tail[b] from pens including pigs with fresh tail lesions.	10 (9:1)	10	5
Pig with mild tail lesion (MTB)	Pig from pens including pigs with fresh tail lesions. Tail is (close to) full length with a fresh wound (redness or blood) or a brownish scab approximately <2 cm in diameter.	13 (8:4)	12	5
Pig with severe tail lesion (STB)	Pig from pens including pigs with fresh tail lesions. Tail has a fresh wound (redness or blood) or a brownish scab. The lesion is approximately >2 cm in diameter or the tail is clearly shortened.	15 (3:11)	13	6

[a] Female to male pig ratio. All male pigs were castrated. The sex was unrecorded for two pigs.

[b] Five of the CTB had very minor signs of lesions on the tail, such as a small, barely visible scab, or a slightly scarred, but fully healed tail tip. These pigs were included as no completely intact tails were available in the pen.

2.3. Sampling procedure

The pigs were between 48 and 62 days of age at the time of sampling. Pigs were sampled between 10.00 and 13.00 by two researchers with experience of saliva sampling in pigs. After identifying suitable pens from the corridor, the sampler entered the pen calmly and when necessary slowly walked through the pen to identify suitable pigs without startling the pigs. If necessary, the sampler squatted down to allow pigs to approach and allow sampling. Straw was used to attract the attention of the pigs if necessary. To avoid any effect of sampling on the biomarkers, only pigs that chewed on the sampling sponge voluntarily were included (i.e. either approached the sampler themselves or allowed to sampler to approach without withdrawing). No animals were restrained for sampling.

Sampling was performed by slightly modifying the methods described in López-Arjona et al. (2020). Pieces of polypropylene sponges were held with metal forceps and gently inserted into the pig's mouth, unless the pig started to chew on its own initiative. The pigs were allowed to chew on the sponge for about 30 s. Once the sponge was clearly wet, it was placed in a Salivette tube (Sarstedt, Germany). Tubes were kept cool and centrifuged at 3000 xg at 4 °C for 10 min. Saliva samples were stored at −80 °C until analysis. Samples were sent for

Table 2
Assay methods used for the different biomarkers.

Biomarker	Assay used	Reference
Oxytocin	Direct competitive AlphaLisa with a monoclonal antibody (oxy-mono)	López-Arjona et al. (2020)
	Indirect competitive AlphaLisa with a polyclonal antibody (oxy-poly)	López-Arjona et al. (2021)
Procalcitonin	Indirect competitive AlphaLisa (polyclonal antibody)	López-Martínez et al. (2022)
Cortisol	Indirect competitive AlphaLisa (monoclonal antibody)	López-Arjona et al. (2020)
Haptoglobin	Direct competitive AlphaLisa (monoclonal antibody)	Contreras-Aguilar et al. (2021)
ADA and its isoenzymes ADA1 and ADA2	Spectrophotometric automated assay (Adenosine Deaminase assay kit, Diazyme Laboratories)	Tecles et al. (2018) Contreras-Aguilar et al. (2020)

analysis in a cold box layered with dry ice to the Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU) at the University of Murcia (Murcia, Spain).

2.4. Biomarker assays

All biomarker assays have been previously described (Table 2).

2.5. Data handling and statistical analysis

All statistical analyses were performed with IBM SPSS v. 28. Biomarkers were checked for normality using Wilk-Shapiro tests and visual estimation. Oxy-mono was found to be non-normally distributed and was thus Log10-transformed to achieve normality.

The difference in biomarkers for pigs with mild (MTB) and severe tail biting lesions (STB) were initially analysed with *t*-tests. These two phenotypes did not differ in any biomarkers and thus were combined for further analysis into a single group of lesioned pigs (LTB).

As it was not possible to find pure control pens in all rooms, some of the control pens also included some pigs with mild lesions (3 pens), healed tail lesions (2 pens), or ear lesions (1 pen). To ensure these pens were still usable as control pens, the biomarkers of pigs from these pens were compared to pigs in the pure control pens with *t*-tests. No differences were observed and thus all control pens were included in subsequent analyses.

As the aim was to compare biomarkers in pigs with tail-biting-related stress, we performed two planned comparisons. First, we compared CC pigs to CTB pigs to assess a possible effect of the tail-biting pen environment. Second, we compared CC pigs to LTB pigs to assess the possible effect of being a victim of tail biting in addition to that of the tail-biting pen environment. Comparisons were performed using separate linear mixed models for each of the biomarkers. Preliminary analyses with *t*-tests showed that there was a sex difference in biomarkers only for oxytocin and cortisol. The models for oxy-mono, oxy-poly, and cortisol thus included phenotype (either CC vs CTB or CC vs LTB) and sex as fixed factors and initially the interaction between sex and phenotype. The interaction was not significant and was thus removed from all final models. Models for all other biomarkers only included phenotype as fixed factor. All models included pen as a random factor. Model residuals were checked for normality. As the model residuals for total ADA and ADA1 when comparing CC pigs with CTB pigs did not show a normal distribution when using original values, these biomarkers were further Log10-transformed and models were rerun with transformed values.

Descriptive statistics for oxy-mono are presented as medians and quartiles from raw data. Other descriptive results are presented as marginal means and standard errors based on model estimations.

To check for correlations between different biomarkers, Pearson's correlations were run separately for all three phenotypes. Log10-transformed data were used for non-normally distributed variables.

P-values <0.05 were considered significant and *P*-values <0.1 as tendencies.

3. Results

It was not possible to identify all phenotypes in all TB pens, and in a few cases more than two LTB pigs were sampled from the same pen. In addition, the sample sizes (Table 3) varied slightly as the amount of saliva was insufficient for analysis all biomarkers. See Table 3 for descriptive data for biomarkers.

The only biomarker that differed between the CC and the CTB pigs was cortisol ($F_{1,37} = 14$, $P < 0.001$) (Fig. 1). Sex was not significant and was thus removed from the final model.

Oxy-poly ($F_{1,20} = 4.0$, $P = 0.06$) and oxy-mono ($F_{1,24} = 4.9$, $P = 0.06$) tended to be higher in CC than in LTB pigs (Fig. 2a and b). For both types of oxytocin, males had higher levels than females (estimated marginal mean 729 (standard error 80) vs 444 (73) ng/mL, $F_{1,59} = 7.8$, $P = 0.007$ and median 3341 (interquartile range 4596) vs 2652 (2291) pg/mL, $F_{1,49} = 4.9$, $P = 0.03$, respectively). PCT concentration tended to be higher in LTB than CC pigs ($F_{1,50} = 3.3$, $P = 0.07$) (Fig. 3a). Cortisol levels did not differ by phenotype ($P > 0.1$) but was higher in male pigs than in female pigs (66 (9.0) vs 46 (8.9) ng/mL, $F_{1,49} = 5.9$, $P = 0.02$). Hp levels were significantly higher in LTB pigs than in CC pigs ($F_{1,33} = 16$, $P < 0.001$) (Fig. 3b). There were no differences between phenotypes in any of the ADA measures (all $P > 0.1$).

Correlations between all biomarkers for the different phenotypes (CC, CTG, and LTB) separately (Table 4a-4c) were mainly positive.

4. Discussion

This study provides additional support for the validity of saliva oxytocin as a measure of stress in pigs. In comparison to the transport

Table 3
Descriptive statistics (mean and standard deviation (SD)) for the measured saliva biomarkers in control pigs in control pens (CC, $n = 30$), control pigs in tail biting pens (CTB, $n = 10$), and lesioned pigs in tail biting pens (LTB, $n = 28$).

	CC			CTB			LTB		
	N	Mean/*median*	SD/*IR*	N	Mean/*median*	SD/*IR*	N	Mean/*median*	SD/*IR*
Oxytocin monoclonal (pg/mL)[a]	28	*3684.2*	*4766.0*	9	*3234.8*	*4263.0*	27	*2644.8*	*2467.0*
Oxytocin polyclonal (ng/mL)	28	647.2	440.1	9	554.2	503.6	27	498.6	319.5
Procalcitonin (ng/mL)	28	3844.6	2847.8	8	3513.3	2416.8	26	5486.7	4019.7
Cortisol (ng/mL)	30	53.2	39.4	10	62.5	60.3	28	47.6	31.1
Haptoglobin (ng/mL)	28	2328.3	1456.7	9	3597.1	1924.9	27	4056.9	1734.2
ADA[b] (U/L)	30	1240.3	1012.7	10	1388.7	717.3	28	1676.2	991.8
ADA[b]1 (U/L)	30	1222.8	1011.2	10	1369.6	718.0	28	1659.8	990.1
ADA[b]2 (U/L)	30	17.6	10.9	10	19.1	11.7	28	16.3	8.2

[a] Monoclonal oxytocin values did not fit a normal distribution and thus median and interquartile range (IR) are given.
[b] Adenosine-deaminase.

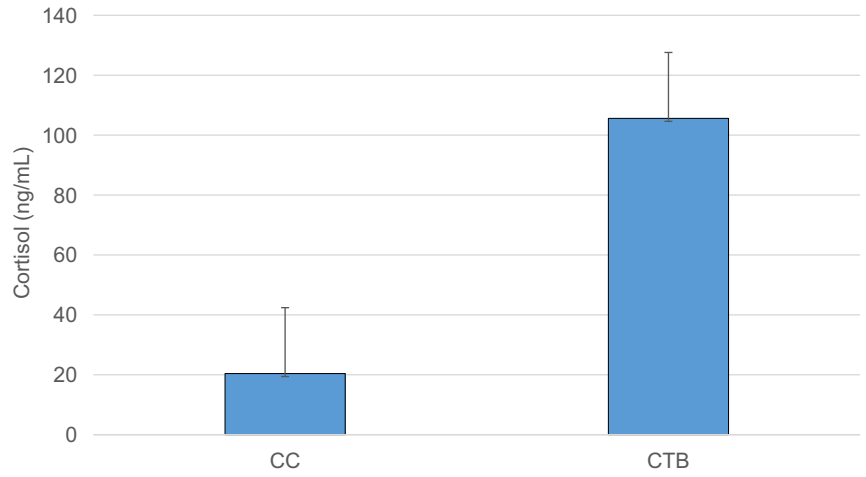

Fig. 1. Estimated marginal means and standard error for cortisol (ng/mL) in control pigs from control pens (CC) and control pigs from tail-biting pens (CTB). The phenotypes differ significantly ($P < 0.001$).

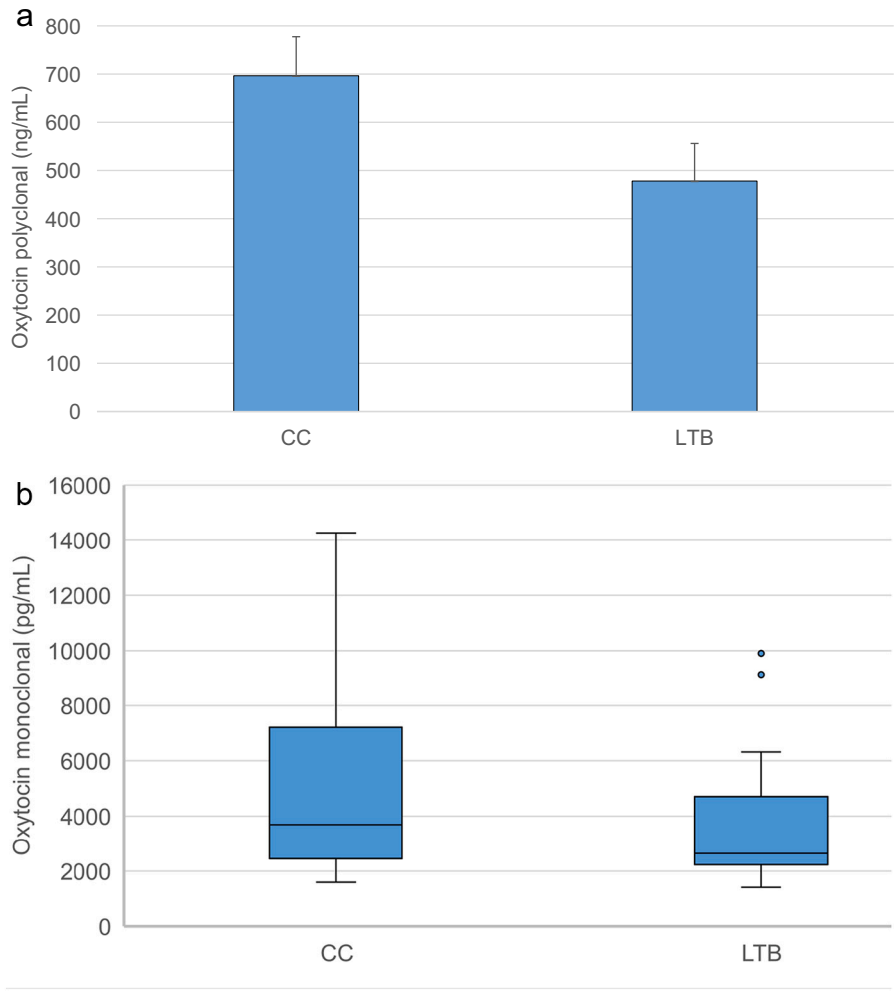

Fig. 2. (a) Estimated marginal means and standard error for polyclonal oxytocin (ng/mL) and (b) boxplot based on original values for monoclonal oxytocin (pg/mL) in control pigs from control pens (CC) and lesioned pigs from tail-biting pens (LTB). The two phenotypes tend to differ for both oxytocin measures ($P = 0.06$ for both).

stress modelled by López-Arjona et al. (2020), this study compared baseline, home pen samples of pigs of different phenotypes. Oxytocin tended to be lower in pigs with tail-biting lesions compared to control pigs in control pens, indicating that these pigs may suffer from more stress, or, alternatively, less positive states.

We found a tendency for lower oxytocin concentration in LTB than CC pigs, while there was no difference in cortisol. Oxytocin and cortisol can be interrelated, with oxytocin suggested as a buffer of stress reactions (Tops et al., 2007; Tops et al., 2012). Oxytocin secretion is related to social situations and affects social bonding (Lee et al., 2009;

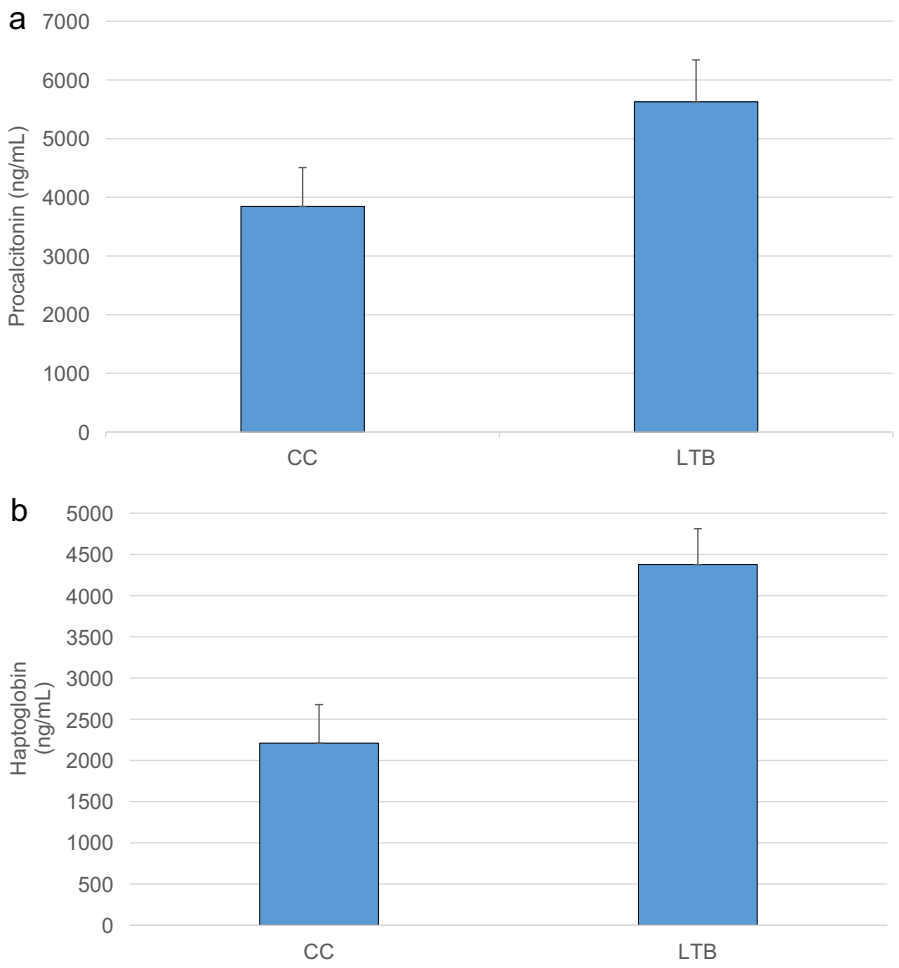

Fig. 3. (a) Estimated marginal means and standard error for procalcitonin (ng/mL) and (b) haptoglobin (ng/mL) in control pigs from control pens (CC) and lesioned pigs from tail-biting pens (LTB). The phenotypes tended to differ for procalcitonin ($P = 0.07$) and differed significantly in haptoglobin levels ($P < 0.001$).

Table 4a
Correlations between biomarkers including controls in control pens (CC) (n = 30). Significant correlations ($P < 0.05$) are indicated by bolding and tendencies ($P < 0.1$) by bolded italics.

		Oxytocin polyclonal (pg/mL)	Procalcitonin (ng/mL)	Cortisol (ng/mL)	Haptoglobin (ng/mL)	ADA[a] (U/L)	ADA[a]1 (U/L)	ADA[a]2 (U/L)
Oxytocin monoclonal (pg/mL)	r_p	**0.86**	**0.61**	**0.50**	**0.56**	**0.39**	***0.38***	**0.47**
	P	**<0.001**	**<0.001**	**0.007**	**0.002**	**0.04**	***0.05***	**0.01**
	n	**28**	**26**	**28**	**28**	**28**	***28***	**28**
Oxytocin polyclonal (ng/mL)	r_p		**0.46**	**0.62**	**0.64**	0.28	0.27	**0.58**
	P		**0.02**	**<0.001**	**<0.001**	0.15	0.17	**0.001**
	n		**26**	**28**	**28**	28	28	**28**
Procalcitonin (ng/mL)	r_p			−0.05	0.26	0.02	0.02	0.26
	P			0.82	0.21	0.92	0.93	0.19
	n			28	26	28	28	28
Cortisol (ng/mL)	r_p				0.34	0.26	0.25	**0.48**
	P				0.08	0.17	0.18	**0.007**
	n				28	30	30	**30**
Haptoglobin (ng/mL)	r_p					0.15	0.14	**0.52**
	P					0.46	0.48	**0.005**
	n					28	28	**28**
ADA[a] (U/L)	r_p						**1.0**	0.15
	P						**<0.001**	0.43
	n						**30**	30
ADA[a]1 (U/L)	r_p							0.14
	P							0.47
	n							30

[a] Adenosine-deaminase.

Table 4b
Correlations between biomarkers including controls in tail biting pens (CTB) (n = 10). Significant correlations ($P < 0.05$) are indicated by bolding.

		Oxytocin polyclonal (pg/mL)	Procalcitonin (ng/mL)	Cortisol (ng/mL)	Haptoglobin (ng/mL)	ADA[a] (U/L)	ADA[a]1 (U/L)	ADA[a]2 (U/L)
Oxytocin monoclonal (pg/mL)	r_p	**0.99**	0.38	**0.93**	0.43	−0.08	−0.82	**0.73**
	P	**<0.001**	0.41	**<0.001**	0.25	0.85	0.83	**0.03**
	n	**9**	7	**9**	9	9	9	**9**
Oxytocin polyclonal (ng/mL)	r_p		0.41	**0.95**	0.50	−0.14	−0.15	**0.76**
	P		0.37	**<0.001**	0.2	0.72	0.71	**0.02**
	n		7	**9**	9	9	9	**9**
Procalcitonin (ng/mL)	r_p			0.6	−0.82	0.31	0.24	0.34
	P			0.12	0.86	0.94	0.96	0.41
	n			8	7	8	8	8
Cortisol (ng/mL)	r_p				0.42	0.1	0.09	**0.81**
	P				0.27	0.79	0.81	**0.004**
	n				9	10	10	**10**
Haptoglobin (ng/mL)	r_p					−0.16	−0.16	0.54
	P					0.69	0.68	0.14
	n					9	9	9
ADA[a] (U/L)	r_p						**1.0**	−0.06
	P						**<0.001**	0.88
	n						**10**	10
ADA[a]1 (U/L)	r_p							−0.07
	P							0.84
	n							10

[a] Adenosine-deaminase.

Table 4c
Correlations between biomarkers including pigs with tail lesions (LTB) (n = 28). Significant correlations ($P < 0.05$) are indicated by bolding and tendencies ($P < 0.1$) by bolded italics.

		Oxytocin polyclonal (pg/mL)	Procalcitonin (ng/mL)	Cortisol (ng/mL)	Haptoglobin (ng/mL)	ADA[a] (U/L)	ADA[a]1 (U/L)	ADA[a]2 (U/L)
Oxytocin monoclonal (pg/mL)	r_p	**0.82**	−0.14	**0.43**	**0.56**	0.13	0.12	**0.63**
	P	**<0.001**	0.52	**0.02**	**0.003**	0.52	0.54	**<0.001**
	n	**27**	25	**27**	**27**	27	27	**27**
Oxytocin polyclonal (ng/mL)	r_p		−0.06	**0.57**	**0.52**	0.33	0.32	**0.73**
	P		0.76	**0.002**	**0.005**	0.10	0.10	**<0.001**
	n		25	**27**	**27**	27	27	**27**
Procalcitonin (ng/mL)	r_p			***−0.39***	0.09	0.06	0.06	−0.06
	P			***0.05***	0.69	0.76	0.76	0.79
	n			***26***	25	26	26	26
Cortisol (ng/mL)	r_p				0.25	0.11	0.11	**0.70**
	P				0.22	0.58	0.59	**0.01**
	n				27	27	27	**27**
Haptoglobin (ng/mL)	r_p					0.11	0.11	**0.40**
	P					0.58	0.59	**0.04**
	n					27	27	**27**
ADA[a] (U/L)	r_p						**1.0**	0.21
	P						**<0.001**	0.29
	n						**28**	28
ADA[a]1 (U/L)	r_p							0.20
	P							0.31
	n							28

[a] Adenosine-deaminase.

Lürzel et al., 2020). Thus, the results suggest that LTB pigs were under more social stress. Alternatively, these results may reflect a decrease of positive states in pigs suffering from tail biting. In addition to the underlying stress behind the outbreak, tail biting causes the victim pigs stress and pain (Munsterhjelm et al., 2013; Sandercock et al., 2019). Oxytocin is often referred to as the 'feel-good' hormone or as an indicator of positive emotions (Mitsui et al., 2011). In addition, oxytocin has analgesic effects (Xin et al., 2017), with lower oxytocin concentrations in humans in pain (Oladosu et al., 2020). However, oxytocin regulation and its effects remain poorly understood (Rault et al., 2017) and further studies are warranted.

CTB pigs did not have different oxytocin levels than CC pigs, although the values were numerically intermediate to the two other phenotypes. However, cortisol was higher in CTB than CC pigs. The results from CTB pigs should be interpreted with caution. First, half of the pigs were actually not pure controls due to practical constraints but had very minor signs of lesions upon close inspection of the tail. Three CTB pigs had a barely visible scab on their tail tip, and two had signs of a very mild previous tail injury, with healed scar tissue at the tail tip. Secondly, as the CTB pig was sometimes the only pig in the pen with an intact tail, it is possible that these pigs were actually biters. We did not systematically try to identify biters. Munsterhjelm et al. (2013) revealed that biters may have an increased stress level, which may explain this result. On the other hand, if the CTB pigs were true 'neutrals' (i.e. neither biters nor victims), one additional explanation may be that pigs change their behaviour to stay neutral during a tail biting outbreak, such as by reducing feed intake (Palander et al., 2013), which may be stressful. Finally, cortisol may not be very informative due to its diurnal variation (Merlot et al., 2011). However, in our study the animals were sampled in a similar diurnal secretion phase.

Consistent with several previous studies (Brown et al., 2016), we observed a correlation between cortisol and oxytocin. However, López-

Arjona et al. (2020) did not find a correlation between cortisol and oxytocin in their transport study. This is consistent with the results of Alley et al. (2019), who observed a correlation only in baseline but not in post stress-treatment values. The very high correlation in especially CTB pigs ($r > 0.9$), which also had higher cortisol levels than CC pigs, suggests differing regulation of the feedback from oxytocin on the HPA axis. A possible dysregulation, seen as correspondingly high or low levels of both oxytocin and cortisol, was shown in connection to post-traumatic stress disorder in humans (Li et al., 2019).

The increased levels of PCT in LTB pigs suggests a potential spread of bacteria from the tail lesions to the bloodstream (Sihvo et al., 2012). In humans, although PCT can identify severely infected ulcers (Jeandrot et al., 2008), PCT can also increase in certain traumas per se (Parli et al., 2018). Therefore, further studies are needed to establish PCT ranges for confirming infection in pigs. In addition, further research should be performed to elucidate the reason of the strong correlation between oxytocin and procalcitonin that appeared in CC pigs only.

The higher Hp in LTB than in CC pigs is not surprising, given that similar results were observed in previous studies (Heinonen et al., 2010; Carroll et al., 2018; Petersen et al., 2002) and Hp is a validated marker of inflammation, trauma, and infection (Cerón et al., 2022). In addition, Hp has been suggested as a biomarker for stress (Salamano et al., 2008). The latter may also explain why Hp levels were numerically higher in CTB when compared with CC pigs and the positive correlation with oxytocin. In addition, there is increasing evidence of ill-health being a risk factor for tail biting (Nordgreen et al., 2020). It cannot be excluded that the pigs in the TB pens were suffering for subclinical illness. Respiratory diseases and lameness are possible health-related risk factors for tail biting (Boyle et al., 2021) and both are linked to an increased level of Hp (Petersen et al., 2002; Gutiérrez et al., 2009).

Although ADA and its isoenzymes did not show significant variation between groups, ADA2 correlated with Hp and also with oxytocin and cortisol especially in pigs from TB pens. This could indicate a relation between ADA and stress, as previously reported in pigs where ADA1 and ADA2 correlated with pain score in lame and prolapsed pigs (Contreras-Aguilar et al., 2019). This link warrants further investigation.

Due to practical constraints, only pigs that voluntarily chewed on the sampling sponge were included. These pigs are potentially bold pigs with a high motivation for exploration. Previous studies have shown that tail-biting behaviour is linked to an explorative phenotype (Ursinus et al., 2014; Haigh et al., 2020). Thus, it is possible that the sampling procedure influenced the results. However, Haigh et al. (2020) did not find a difference in baseline cortisol levels between bold and shy pigs. Further, the procedure was the same for all phenotypes included in the study, which should minimize the risk of bias.

This was a first pilot study to explore the relationship between tail-biting phenotype and oxytocin and procalcitonin and the number of animals used was limited by practicalities. However, due to the high variability of these biomarkers, a larger number of animals should be included in future studies to corroborate these pilot findings. Based on post hoc power analyses, the sample size to achieve significant results for oxytocin and procalcitonin would be approximately 50–70 animals per phenotype. Furthermore, we did not perform blood cultures to verify if the higher concentrations of PCT in LTB pigs was linked to sepsis. Future studies should test the possibility of using biomarkers related to stress as predictors of tail-biting outbreaks, and for identifying pigs in which the lesion caused a systemic bacterial infection. In addition, repeated individual measurements of the biomarkers would be recommended especially for monitoring purposes and for better understanding the co-regulation of the different biomarkers.

5. Conclusions

The results of this study indicate that oxytocin and procalcitonin may be potential biomarkers of stress and sepsis in tail biting in pigs. In addition, these results provide further support for the link between stress and tail biting and previous data on tail-biting lesions being potential routes for systemic spread of bacteria.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article 8 (published):

Measurement of Calprotectin (S100A8/A9) in the Saliva of Pigs: Validation Data of A Commercially Available Automated Assay and Changes in Sepsis, Inflammation, and Stress

animals

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Article

Measurement of Calprotectin (S100A8/A9) in the Saliva of Pigs: Validation Data of A Commercially Available Automated Assay and Changes in Sepsis, Inflammation, and Stress

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Simple Summary: Calprotectin (CALP, S100A8/A9) is a calcium and zinc-binding protein involved in inflammation that has a wide range of proinflammatory functions, such as cytokine production and regulation of leukocyte adhesion, migration, and phagocytosis. The objective of this study was to validate a commercially available assay for the measurement of CALP in the saliva of pigs and study the variations of this analyte due to sepsis, non-septic inflammation, and stress. The assay showed adequate precision and accuracy for the measurements of CALP in the saliva of pigs. In addition, this protein showed significant increases in the saliva of pigs with sepsis as well as after a stressful situation in our experimental conditions, being the increase in the stress of lower magnitude than in sepsis. Based on these results, CALP can be measured in the saliva of pigs and could be a potential biomarker of health and welfare in this species.

Abstract: Calprotectin (CALP, S100A8/A9), also named myeloid-related protein 8/14, is a dimer complex of S100A8 and S100A9 that belongs to the S-100 protein family. It is involved in inflammation and has a wide range of proinflammatory functions, such as cytokine production and regulation of leukocyte adhesion, migration, and phagocytosis. In humans, CALP traditionally can be measured in faeces, serum, and saliva as a biomarker of inflammation and sepsis. The objective of this study was to validate an automated assay for CALP measurements in the saliva of pigs, having the advantage of the use of a non-invasive sample that is easy to collect. The assay was precise and accurate. CALP in saliva measured by this assay showed significant changes depending on the hour of the day. It also showed significant increases in the saliva of pigs after the administration of lipopolysaccharide (LPS), and showed a rise, although with increases of lower magnitude, after a stressful stimulus. Further studies should be made to gain knowledge about the possible practical applications of the measurements of CALP in the saliva of pigs as a biomarker to evaluate the animals' health and welfare.

Keywords: S100A8/A9; calprotectin; automated assay; pig; saliva; stress; sepsis

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1. Introduction

Calprotectin (CALP, S100A8/A9), also named myeloid-related protein 8/14, is a dimer complex of S100A8 and S100A9 which belongs to the S-100 protein family [1]. It is a calcium and zinc-binding protein involved in inflammation and has a wide range of proinflammatory functions, such as cytokine production and regulation of leukocyte adhesion, migration, and phagocytosis [2].

Currently, there is an increasing interest in the use of saliva as a biological sample since it has the advantage of being a non-invasive sample that is easy to collect. This is highly relevant in pigs, a species in which blood sampling is difficult, highly stressful, and painful for the animal. The collection of saliva can be easily made by farm personnel, allowing a more frequent analysis and monitoring to take place at the farm level. Currently, one of the main uses of saliva is for the diagnosis and detection of infectious diseases, but saliva can also be used to measure biomarkers that can provide information on stress, inflammation, immune response, and redox homeostasis [3]. Therefore, saliva contains a source of analytes with the potential to assess the effect of different husbandry conditions and also to evaluate the homeostasis in swine.

In humans, CALP traditionally has been measured in faeces for the detection of inflammatory bowel disease and in serum as a biomarker of inflammation and sepsis [4]. However, it can also be quantified in saliva, where CALP has been reported to be increased in patients with active inflammatory bowel disease, suggesting that intestinal inflammation leads to increases in CALP in this sample type [5]. In pigs, CALP protein levels have been measured in faeces, increasing in animals with colitis [6–8]. In addition, variations in gene expression for calprotectin have also been found in the ileum after oral vaccination against *E. coli* [9]. However, to the authors' knowledge, there are no reports about the measurement of CALP in saliva, despite the advantages that this sample type has.

The objectives of this report were to perform an analytical validation of an automated commercially available assay for the measurement of CALP in the saliva of pigs and to determine if it is influenced by possible variations due to the sampling time during the day. In addition, the variations of this analyte due to sepsis, non-septic inflammation and stress were studied. For this purpose, the CALP concentration was measured in the saliva of pigs with three different conditions: sepsis experimentally induced by inoculation with lipopolysaccharide (LPS), non-septic inflammation induced by turpentine injection and a situation of stress such as staying at the lairage for 4 h after arrival at the slaughterhouse. In the models of LPS and turpentine, the correlation between values of CALP in saliva and CALP and C-reactive protein (CRP) in serum was studied. It is expected that this information will contribute to a better knowledge of the pathophysiology of this protein and its possible use as a biomarker of these conditions in saliva.

2. Materials and Methods

2.1. Assay

Salivary CALP was determined by the BÜHLMANN fCal Turbo® assay (BÜHLMANN, Laboratories AG, Switzerland) which is an immunoturbidimetric assay using polystyrene nanoparticles coated with polyclonal anti-CALP antibodies. The assay was initially calibrated with serial dilutions of a control material with a known concentration of CALP (Gentian, Moss, Norway) (Calibrator A). Then, a secondary calibrator (Calibrator B) consisting of a pool of saliva samples, in which the CALP concentration was determined with Calibrator A, was used for the complete validation and subsequent sample analysis.

The CALP assay was validated for use in porcine saliva samples using aliquots of the saliva of pigs of the sepsis and non-septic inflammation experiment (Section 2.3). The validation of the assays was performed as follows based on previous procedures [10]:

- Precision: the intra- and inter-assay coefficients of variation (CVs) were assessed using saliva samples with high and low CALP concentrations.
- Accuracy: It was indirectly studied by evaluating linearity after serial dilutions with ultrapure water of saliva and samples with a high level of CALP. Additionally, recovery

studies were made to see if there was a matrix effect in the determinations. For this, purified CALP (control material from Gentian, Moss, Norway) was used to spike saliva samples to reach three different CALP concentrations.

- The lower limit of quantification (LLQ): it was calculated as the lowest CALP concentration that the assay was able to determine with an intra-assay CV < 20%.
- Limit of detection (LD): based on the lowest concentration of CALP that the assays can distinguish from a specimen of zero value (ultrapure water), calculated as a mean value plus 3 standard deviations of 12 replicate determinations.

2.2. Evaluation of the Sampling in the Daytime

To determine the effect of the time of sampling, surplus saliva samples from 20 male large white pigs aged 5 months from a commercial farm stored from a previous study were used [11]. In these pigs, saliva was sampled at 8 a.m., 12 a.m., 4 p.m., and 8 p.m. within the same day. In this experiment (and in all trials of the report), saliva was collected using Salivette tubes (Salivette, Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany). Pigs chewed a sponge for 1 min. Each sponge was then placed in a Salivette tube and stored on ice until arrival at the laboratory. At the laboratory, the tubes were centrifuged at 3000× *g* for 10 min. Saliva samples were kept frozen at −80 °C until analysis.

2.3. Experimental Sepsis and Non-Septic Inflammation Induction

To evaluate the changes in CALP concentration caused by sepsis and non-sepsis inflammatory conditions, samples stored from a previous study were used [12]. A total of 15 growing male pigs, in the mid-fattening period, from the University of Murcia Farm, were included in this study. They had water ad libitum and a balanced diet and had a minimum space of 0.65 m^2 per animal (Council Directive 2001/88/CE of 23 October 2001) with a mean temperature of 24 ± 2 °C. The pigs were 14 weeks old and their median weight was 51.5 kg (interquartile range 48–53 kg).

The pigs were adapted to the experimental conditions for a week and then randomized and divided into three groups of 5 animals each. A control group (n = 5) received saline treatment (2 mL) by an intramuscular route. An LPS group (n = 5) received a single dose of 30 µg/kg LPS from Escherichia Coli (LPS; O55:B5, Sigma-Aldrich, St. Louis, MO, USA) in sterile saline solution by intramuscular injection. A turpentine (TURP) group (n = 5) received 8 mL of TURP (oil of turpentine purified, Sigma–Aldrich) by two 4 mL subcutaneous injections in each front flank per animal. The injections were completed between 8 and 9 a.m.

Saliva and blood samples were obtained 24 h before (baseline) the saline, LPS, or TURP injections and at 6, 24, and 48 h after the treatments. Basal, 24 h, and 48 h samples were obtained at 8 a.m. Saliva was collected and processed as indicated in Section 2.2. Blood samples were collected by venepuncture in an EDTA and plain tubes and serum were separated and stored at −80 °C.

2.4. Stress Situation

A total of 13 male pigs at the end-fattening period (5–6 months of age and mean body weight 105.2 ± 7.3 kg) were included in this study. They were from a farm in Southern Spain, where animals were housed in groups of 14 animals per pen (a minimum space of 0.65 m^2 per animal) and given ad libitum access to a balanced diet and water. The pigs were transported to a commercial slaughterhouse at 15 km from the commercial farm. Transportation was undertaken during the spring of 2022, between 9 and 10 a.m., under commercially accepted conditions. The animals were unloaded on arrival at the slaughterhouse and placed in a lairage area (10 animals per pen) with free access to water. Saliva samples were collected at arrival at the slaughter on the day of transport (T0) (approximately at 10 a.m.) and 4 h after the transport (approximately at 2 p.m.) (T4). The transport of animals was according to the recommendations described in Directive 2001/88/EC, 2001 and Directive 2001/93/EC, 2001.

Saliva samples were obtained as described in Section 2.2. Salivary cortisol concentrations of each animal were measured in these samples with a validated assay [13].

All the experimental procedures of this study were approved by the Ethical Committee on Animal Experimentation (CEEA) of the University of Murcia (A13220196; approval date: 4 March 2021) according to the European Council Directives considering the protection of animals used for experimental purposes. In addition, this study complies with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for the care and use of animals.

2.5. Statistical Analysis

GraphPad Prism software Inc. (GraphPad Prism, version 8 for Windows, Graph Pad Software Inc., San Diego, CA, USA) was used to analyse the data. The D'Agostino and Pearson test was used to evaluate the data distribution giving a nonparametric distribution in all analyses. To analyse the effect of the day on salivary CALP concentrations and the effect of treatment in the LPS, TURP and saline (control) group, the Friedman test was performed, followed by Dunn's multiple comparison tests to compare the groups over time. The comparison between the groups of pigs after transportation was performed by using the Wilcoxon matched pairs signed rank test. Correlations between salivary CALP and serum CALP and CRP (data of serum CRP in these animals have been reported in a previous study [12]) were analysed through the non-parametric Spearman correlation test. Moreover, in the stress situation, the correlation between salivary CALP and cortisol was evaluated by the same test. A correlation was strong when the correlation coefficient was $\geq$0.7. The results were considered significant if p-values were <0.05.

3. Results

3.1. Saliva Calprotectin Assay Validations

The evaluation of precision showed mean intra- and inter-assay CVs of 3.50 and 4.79% for salivary CALP (Table 1). In the accuracy assessment, we observed recovery rates ranging from 110% to 116.7% for salivary CALP measurements (Table 2). Furthermore, the serial dilution of saliva samples with a high concentration of CALP showed linear regression equations with a coefficient of correlation close to 1 (Supplementary Material: Figure S1). The LLQ was set at 0.01 mg/L for salivary CALP, and the LD of the assay could not be calculated since all measurements with ultrapure water gave a value of zero.

Table 1. Precision study of the salivary calprotectin assays. (SD, standard deviation; CV, coefficient of variation).

Method	Comparison	Samples	Mean (mg/L)	SD (mg/L)	CV (%)
Saliva	Intra-assay	High	1.54	0.03	2.14
		Low	0.55	0.02	4.86
	Inter-assay	High	1.67	0.04	4.26
		Low	0.43	0.03	6.23

Table 2. Recovery study of the calprotectin assay in saliva.

% Analyte		Expected (mg/L)	Observed (mg/L)	Recovery (%)
100	0	0.72	0.72	100
75	25	0.66	0.60	110
50	50	0.54	0.48	112.5
25	75	0.42	0.36	116.7
0	100	0.24	0.24	100

3.2. Evaluation of the Sampling Time on the Day

The CALP concentrations in saliva showed a tendency to decrease during the day, being these changes significantly at 12 p.m. (median = 0.07 mg/L, range = 0.03–0.30 mg/L)

and 8 p.m. (median = 0.07 mg/L, range = 0.03–0.72 mg/L) if compared with values at 8 a.m. (median = 0.21 mg/L, range = 0.01–0.96 mg/L) (Figure 1).

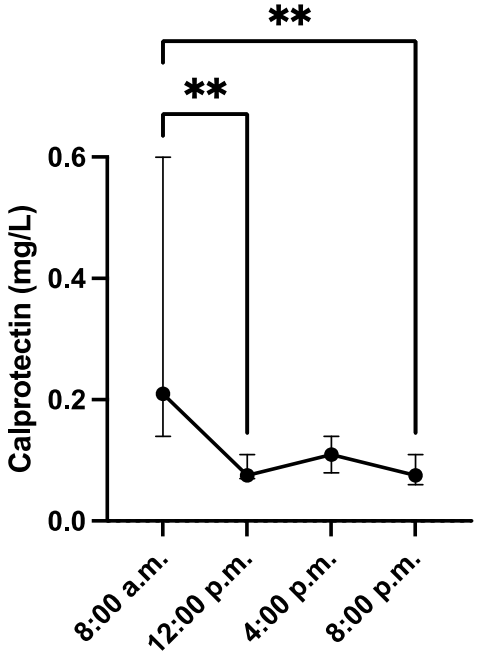

Figure 1. Results of salivary calprotectin concentrations in 20 male pigs obtained at different hours of the day. ** *p*-value < 0.01. Dots are representing median values and whiskers the 95% CI.

3.3. Experimental Sepsis and Non-Septic Inflammation Induction

In saliva, the CALP levels were significantly higher in pigs at 24 h after LPS injection (median = 1.26 mg/L, range = 0.54–1.32 mg/L) compared to basal (pre-treatment) levels (median = 0.18, range = 0.06–0.24 mg/L) (p = 0.005), being reduced to nearly basal levels at 48 h (Figure 2). In the case of the administration of TURP, a tendency of increase was observed after the inoculation, but the changes were not significant. The effect of saline injection did not cause any variation in salivary calprotectin levels.

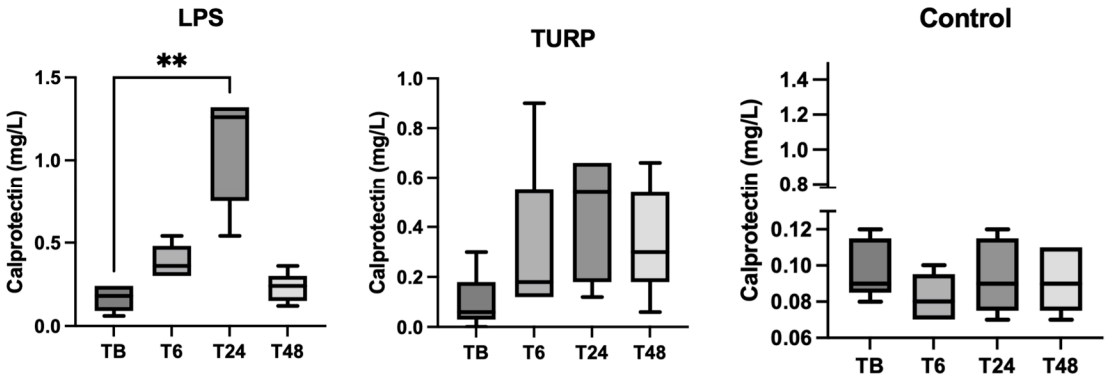

Figure 2. Changes in the salivary calprotectin concentrations of pigs after lipopolysaccharide (LPS), turpentine (TURP) or saline (control) injection. The plots show medians (line within box), 25th and 75th percentiles (boxes) and min and max values (whiskers). Asterisks indicate a statistically significant difference: ** p < 0.01.

In serum, pigs subjected to LPS injection showed a significant increase in CALP concentrations at 6 h (median = 0.17 mg/L, range = 0.09–0.36 mg/L) compared with basal values (median = 0.02 mg/L, range = 0.01–0.03 mg/L) (p = 0.01). In the TURP group, there

were no significant changes in the pairwise comparison although a tendency of increase at 6 h and 24 h compared with the control group was observed (Figure 3).

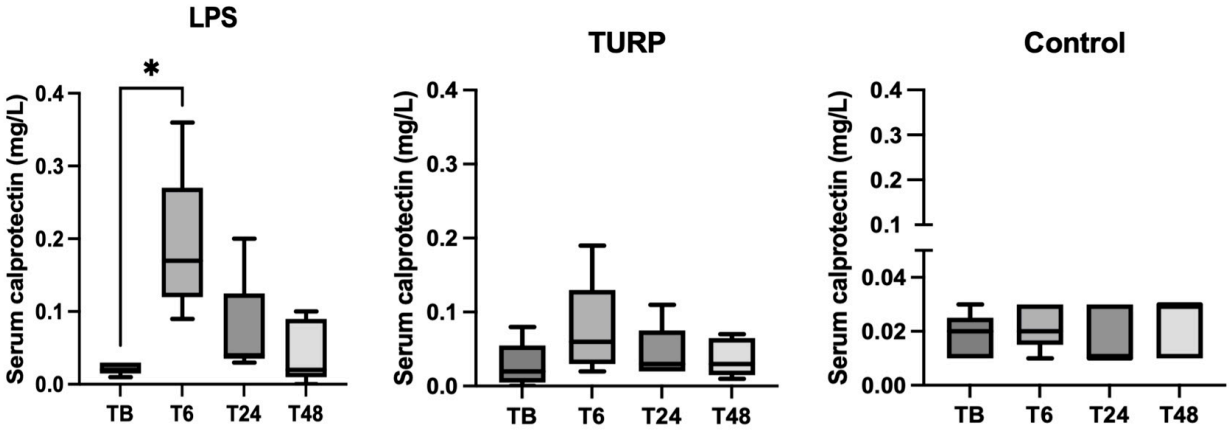

Figure 3. Changes in the serum calprotectin concentrations of pigs after lipopolysaccharide (LPS), turpentine (TURP) or saline (control) injection. The plots show medians (line within box), 25th and 75th percentiles (boxes) and min and max values (whiskers). * *p*-value < 0.05.

The correlation study showed that salivary and serum CALP concentrations were positively correlated (r = 0.283, $p = 0.02$). In addition, serum CRP concentration values were significantly positively correlated with both salivary (r = 0.665, $p = 0.01$) and serum CALP concentrations (r = 0.418, $p < 0.001$). All these correlations were below 0.7 which is considered the threshold for a strong correlation.

3.4. Stress Situation

The concentrations of CALP were significantly higher in pigs 4 h after arrival at the slaughterhouse (median = 0.15 mg/L, range = 0.06–0.78 mg/L) compared with basal values (median = 0.09 mg/L, range = 0.02–0.32 mg/L) ($p = 0.002$) (Figure 4).

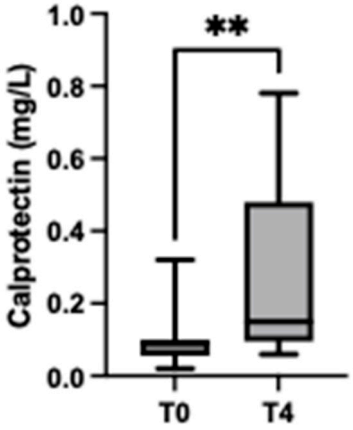

Figure 4. Concentrations of calprotectin in pigs at the arrival to the slaughterhouse (T0) and 4 h after transportation (T4). The plots show medians (line within box), 25th and 75th percentiles (boxes) and min and max values (whiskers). ** *p*-value < 0.01.

Values of salivary CALP showed a significant positive but weak correlation with salivary cortisol concentrations in the pigs included in this comparison (r = 0.396, $p = 0.04$)

4. Discussion

In this report, the validation of an immunoturbidimetric assay for CALP in porcine saliva which allows its automated quantification was performed. The assay was found to be

valid for use in non-invasively collected saliva from pigs. There are various commercially available kits for the detection of CALP in faeces and serum, from ELISAs to point-of-care assays. This assay was selected for validation because it has also been previously described to measure CALP in pig faeces, and therefore it has already been shown to be able to detect CALP in this species. Furthermore, it is an automated assay suitable for veterinary clinical pathology laboratories [6]. This assay uses polystyrene nanoparticles coated with polyclonal antibodies, which form complexes with CALP that can be detected by light absorbance using automated clinical chemistry analysers, therefore it is a rapid assay allowing high sample throughput.

Two modifications were made in this assay. One was the use of a solution of purified human CALP as Calibrator A to initially measure in saliva samples of pigs. With this calibrator, the units for the concentration of CALP in saliva were assessed in mg/L instead of μg/g as is the concentration of the original calibrator of the kit. The second modification was the calibration of this assay with Calibrator B, which consists in a pooled saliva sample with a known concentration of CALP, in order to reduce the possible matrix effect. Overall, the modified assay of our study was precise and linear in saliva, being in line with the previous report in which this assay could detect CALP in the faeces of pigs [6].

The increases after LPS administration found in our study indicate that CALP increases in sepsis, which is in line with the findings in humans where increases in this protein in serum have been found in patients with sepsis. This allows early diagnosis of sepsis on intensive care unit (ICU) admissions in adults [4] as well as infants [14], and is considered to be a tool that can aid timely sepsis management reducing mortality rates and avoiding unnecessary antibiotic treatment, thus improving antibiotic stewardship. The increases found in sepsis in pigs were higher than in the non-sepsis inflammatory condition. This would agree with previous reports in humans which found higher values in sepsis compared with patients with non-septic inflammation [15]. Further studies with a larger number of individuals would be recommended to evaluate the ability of CALP to differentiate between these two processes.

In our study, significant CALP increases in both pigs' saliva and serum after LPS administration were detected. The values of CALP in serum were lower than in saliva in agreement with previous reports in humans [5,16], so a possible production of this protein in saliva could be hypothesised, as it has been described in mice [15]. This could also be a reason for the weak correlation between the concentrations of saliva and serum of CALP in our experimental conditions. Peak increases in serum were earlier than in saliva, and further studies should be undertaken to elucidate the mechanism involved, and also to evaluate which sample type could be more sensitive to detect septic conditions. In general, the values of CALP in serum were small with the absorbances of the assay at the low end of the range; therefore, in the future, the use of other CALP assays could be explored that could generate higher assay signals with this sample type.

An increase in the values of CALP was found after a situation of stress consisting of a 4-h stay at the slaughterhouse. This stress is due to new situations that face the pigs at the slaughterhouse, with various stressful stimuli such as strange sounds and mixing with unfamiliar pigs [17]. Previous reports have indicated that stress can increase faecal CALP in humans, suggesting that this increase could be related to the regulation of the immune system by stress [18], while other reports have associated these increases with the activation of inflammatory processes in the gut that occurs in stressful conditions [19,20]. The increases found in our study in the stress due to pre-slaughter lairage were lower than those found in sepsis, however further studies with other types of stress should be performed to evaluate if in some cases the increases could be high enough to mask a septic situation.

When the assay was applied for the measurement of saliva CALP at different times of the day, the highest values were found at 8 a.m. Changes in the values of some analytes in the saliva of pigs depending on the hour of the day in which the sample is collected have been previously described [11]. In the experiment of septic and non-septic inflammation

induction of this report, samples were obtained at 8 a.m. for the pre-treatment basal as well as the 24 h and 48 h post-treatment samples, whereas the sample collected at 6 h was obtained at 2 p.m. In this case, the magnitude of the change in healthy animals between 8 a.m. and 2 p.m. was not so pronounced as the increases found in the LPS-injected pigs. In the trial of stress induction, it could be postulated that a decrease in calprotectin concentrations would occur between 10 a.m. and 2 p.m. in animals without stress and therefore reinforces the increase found in our study at 2 p.m. after the stressful condition. In any case, sample collection time should be considered an important factor during practical processing, and it would be recommended for the analysis of calprotectin in saliva, if possible, to take the samples at the same time of day. In addition, the study of the possible variations of CALP in saliva during different times of the day in animals with sepsis or different diseases and stress conditions should be assessed to identify if the pattern found here for healthy pigs is also followed in pigs with such conditions.

This study has some limitations. It is important to point out that the results here have been obtained with a specific assay and other assays could provide different values for CALP, as have been reported in humans using different assays such as enzyme-linked immunoassays, automated fluoroimmunoassays, immunochromatographic tests, chemiluminescent or immunoturbidimetric assays [21–23]. Overall, each assay should be independently validated from an analytical and clinical point of view before use in each species under investigation. Additional studies should be made to elucidate the influence in saliva CALP of possible sources of variation such as breed, age, season, or productive condition. Moreover, the trials of sepsis and non-septic inflammation induction were performed with a limited number of animals and should be confirmed in a larger number of individuals. Furthermore, the evaluation of the potential application of CALP in saliva as a biomarker in different diseases within a large population of animals should be performed. In addition, it should be determined if using porcine CALP as a standard could increase its diagnostic value in pigs, as has been reported with other assays, such as the CRP assay in serum when also using kits designed to measure it in humans [24].

5. Conclusions

It can be concluded that CALP can be measured in the saliva of pigs with the assay evaluated in this study and that its concentration showed variations depending on the time of the day in which the sample was obtained. In addition, it was found that CALP increases in the saliva of pigs with sepsis showing, in our experimental conditions, increases of higher magnitude than in pigs with non-septic inflammation. Finally, this protein increases after a stressful situation consisting of a stay of 4 h in lairage at the slaughterhouse, although these increases were of lower magnitude than those in sepsis. Further studies should be made to gain knowledge about the possible practical applications of the measurements of CALP (S100 A8/A9) in the saliva of pigs as a biomarker to evaluate the animals' health and welfare.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13071190/s1, Supplementary Figure S1. Linearity under the dilution of saliva calprotectin assay.

Author Contributions: Conceptualization, S.M.-S., J.J.C., D.E. and A.M.-P.; methodology, J.J.C., P.D.E. and A.M.-P.; software, A.M.-P.; validation, M.J.L.-M., A.O.-B., M.L.-A., D.E. and A.M.-P.; formal analysis, M.J.L.-M., J.J.C. and A.M.-P.; investigation, S.M.-S., J.J.C., G.R., S.M.-M., F.T. and D.E.; resources, G.R., S.M.-M., E.G.M., P.D.E. and D.E.; data curation, J.J.C., and A.M.-P.; writing—original draft preparation, M.J.L.-M., J.J.C. and A.M.-P.; writing—review and editing, M.J.L.-M., S.M.-S., J.J.C., A.O.-B., G.R., M.L.-A., S.M.-M., E.G.M., P.D.E., F.T., D.E. and A.M.-P.; visualization, J.J.C., S.M.-S., D.E. and A.M.-P.; supervision, J.J.C. and A.M.-P.; project administration, J.J.C. and P.D.E.; funding acquisition, S.M.-S., J.J.C., P.D.E., D.E. and A.M.-P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All the experimental procedures of this study were approved by the Ethical Committee on Animal Experimentation (CEEA) of the University of Murcia (A13220196; approval date: 4 March 2021) according to the European Council Directives considering the protection of animals used for experimental purposes. In addition, this study complies with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for the care and use of animals.

Informed Consent Statement: Informed consent was obtained from all the owners whose pigs were involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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Objective 4

Development and validation of new assays to diagnose sepsis: procalcitonin and presepsin.

Article 9 (published):

Measurement of procalcitonin in saliva of pigs: a pilot study

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RESEARCH ARTICLE Open Access

Measurement of procalcitonin in saliva of pigs: a pilot study

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Abstract

Background: Procalcitonin (PCT) is a widely used biomarker of sepsis in human medicine and can have potential applications in the veterinary field. This study aimed to explore whether PCT could be measured in the saliva of pigs and whether its concentration changes in sepsis. Therefore, a specific assay was developed and analytically validated, and changes in PCT concentration were evaluated in two conditions: a) in an experimental model of sepsis produced by the administration of lipopolysaccharide (LPS) to pigs ($n = 5$), that was compared with a model of non-septic inflammation induced by turpentine oil ($n = 4$), and b) in healthy piglets ($n = 11$) compared to piglets with meningitis ($n = 20$), a disease that usually involves sepsis and whose treatment often requires large amounts of antibiotics in farms.

Results: The assay showed coefficients of variation within the recommended limits and adequate linearity after serial sample dilutions. The method's detection limit was set at 68 µg/L, and the lower limit of quantification was 414 µg/L. In the LPS experiment, higher concentrations of PCT were found after 24 h in the animals injected with LPS (mean = 5790 µg/L) compared to those treated with turpentine oil (mean = 2127 µg/L, $P = 0.045$). Also, animals with meningitis had higher concentrations of PCT (mean = 21515 µg/L) than healthy pigs (mean = 6096 µg/L, P value < 0.0001).

Conclusions: According to these results, this assay could be potentially used as a tool for the non-invasive detection of sepsis in pigs, which is currently a topic of high importance due to antibiotic use restriction.

Keywords: Bacterial infections, Biomarkers, LPS, Meningitis, Porcine, Procalcitonin, Saliva, Sepsis, Turpentine oil

Background

Procalcitonin (PCT) is the 13kDa precursor of calcitonin, a hormone with a metabolic role in calcium homeostasis [1, 2]. Almost all PCT is converted into calcitonin in healthy humans, and thus PCT concentrations are in low values in blood [3]. However, in sepsis, PCT is released massively into the bloodstream, and concentrations can rise thousands of fold compared to the physiological values [2, 4–6]. Therefore, its measurement in the blood is widely used in human medicine to diagnose and monitor sepsis and guide antibiotic treatment in bacterial infections, which could be particularly important in the fight against antibiotic resistance [7, 8]. However, there is limited information on the potential applications of this biomarker in veterinary medicine. Particularly in pigs, few studies have been conducted evaluating PCT, and in all cases, this analyte has been measured in serum [5, 9–12].

Saliva is increasingly being used as a diagnostic fluid in animals due to its non-invasive nature and ease of collection by non-specialist personnel. In pigs, saliva is a much more welfare-friendly choice because it avoids the high

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stress that causes blood collection due to restraining. To date, it has been reported in humans that PCT can be measured in this sample type and may have the potential for detecting sepsis [13]. However, to the authors' knowledge, PCT has not been measured in the saliva of any other animal species.

This study aimed to investigate if PCT could be analysed in the saliva of pigs and whether its concentration would change in situations of sepsis. For that purpose, the objectives of this study were to develop and validate a specific assay for the measurement of PCT in pig saliva and to evaluate changes in its concentration in two different situations: a) in an experimental model of sepsis by the administration of lipopolysaccharide (LPS) to pigs, which was compared with a model of non-septic inflammation induced by turpentine oil and b) in pigs from a commercial farm with meningitis, which is a condition associated with sepsis and whose treatment is often related to the use of large amounts of antibiotics [14–16]. This article reports the analytical validation of the new assay developed for the measurement of PCT in saliva and the values of PCT in pigs in the different situations above described.

Results

Optimisation of the method

The optimal concentration of reagents was 4.5 nM for biotinylated PCT, 15 µg/ml for acceptor beads, and 15 µg/ml for donor beads. This combination showed the higher magnitude of signal change before reaching the reaction equilibrium, as well as the maximum buffer signal obtained, and the higher buffer/protein ratio. The final protocol is shown in Fig. 1, and the schematic picture of the AlphaLISA reaction for procalcitonin detection is displayed in Fig. 2.

Analytical validation of the salivary PCT assay

The intra-assay variation showed CVs of 15.59% and inter-assay CVs of 18.19%. The assay also showed adequate linearity after serial sample dilutions, both with a coefficient of determination of $R^2 = 0.99$, as represented in Fig. 3. The mean spike recovery test was 88%, between the recommended limits (80–120%) in all cases. The method's LOD was set at 68 µg/L, and the LLOQ at 414 µg/L.

Changes in salivary PCT after the LPS model

One hour after the injection of LPS, all animals from this group started to show lethargy, increased respiratory rate and depression during approximately 7 h. In addition, one of the animals presented vomiting, and another animal had diarrhoea. The median rectal temperature of the animals after 6 h of the administration of LPS was 41.3ºC.

In the turpentine oil group, the animals showed mild signs of discomfort for several hours but remained alert and active. The median rectal temperature of the animals after 6 h of the administration of turpentine oil was 39.9ºC.

In the multiple comparisons test, higher concentrations of PCT were found at T24 in the animals injected with LPS (mean ± SD = 5790 ± 2060 µg/L) compared to those treated with turpentine oil (mean ± SD = 2127 ± 1365 µg/L), $P = 0.045$. The results are graphically represented in Fig. 4.

Changes in salivary PCT in meningitis

Animals with meningitis had as most frequent symptoms ataxia, anorexia, lateral recumbency, and paddling and a median rectal temperature of 40.5ºC (39.6–40.7ºC, interquartile range). The pigs with meningitis had higher concentrations of PCT (mean = 21515 ± 13289 µg/L) than healthy pigs (mean = 6096 ± 3976 µg/L), with P value < 0.0001. The concentrations of PCT in animals with meningitis vs healthy group are represented in Fig. 5.

Discussion

This study describes the quantification of PCT in the saliva of pigs for the first time. The assay used was a competitive immunoassay, which has the advantage of detecting antigens regardless of their size, making them helpful for quantifying low molecular weight proteins [17]. This assay showed intra and inter-assay imprecision lower than 20%, which is considered the generally accepted limit [18] and also showed high correlation coefficients and good linearity and spike recovery in serially diluted saliva samples. Therefore, it could be used to measure PCT in porcine saliva. This assay is specific for pigs, and this is important since the homology between human and porcine PCT is low [19], which could explain why human ELISA kits cannot detect in some studies PCT concentrations in pigs [5].

The additional advantage of this assay is the use of AlphaLISA technology, which has shorter incubation times than ELISA and does not need any washing step. In addition, it requires a minimum amount of sample (5 µL) for the analysis, which in the case of pig saliva is especially useful in sick animals, in which it is frequent to obtain small amounts of saliva samples.

The concentrations of PCT in healthy and septic pigs found with this immunoassay were higher than those described in human blood. As this is the first time that PCT has been measured in the saliva of pigs, there are no established reference ranges described in this species and sample. Higher PCT values than humans have been observed in other veterinary species such as horses

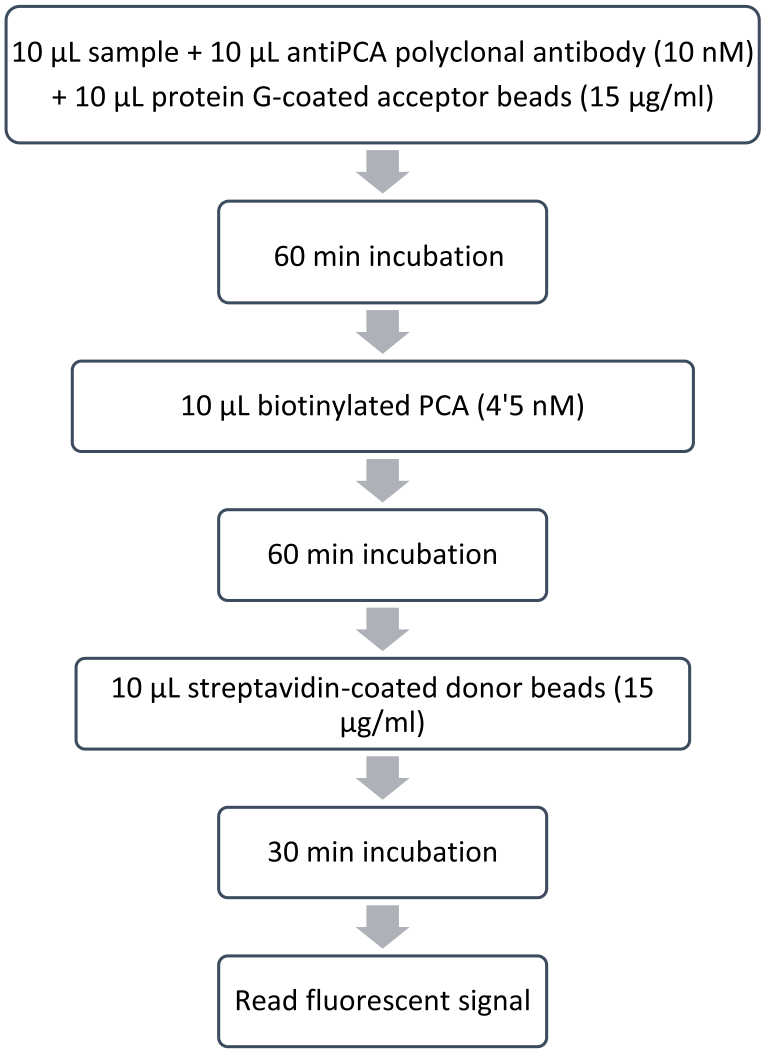

Fig. 1 AlphaLISA protocol for PCT measurement in the saliva of pigs

[20]. Although further studies are needed to understand the reason for these different concentrations, one factor could be the influence in circulating PCT of the species-specific quantities of gram-negative bacteria present in the normal intestinal flora [21, 22]. This factor could explain that in previous reports in horses [23] and our study in pigs, basal concentrations of PCT were detectable, whereas, in humans, basal concentrations of PCT are usually undetectable.

Also, the different magnitude of concentration observed could be due to the type of biological sample or the immunoassay used. For example, in human saliva, two-fold higher concentrations of PCT have been observed compared to serum [13]. Additionally, the antibody used in the assay and its possible affinity to different conformations or states of PCT could lead to differences in immunoassays, as reported with other molecules such as oxytocin [24]. Moreover, PCT is a precursor of the Calcitonin Gene Family of Peptides, which means that other peptides very similar to PCT are eventually released into the bloodstream and could be easily detected by some assays. These peptides have different biological functions, but some share some similarities with PCT, like its possible increase in sepsis, such as adrenomedullin and calcitonin [25, 26].

The experimental model with LPS was used to test the ability of the immunoassay to detect different concentrations of PCT, since higher concentrations of this peptide have been observed in studies with animals treated with LPS [4, 27]. Increases in PCT were also detected in the group treated with turpentine oil, which produces a non-septic inflammation. However, these increases

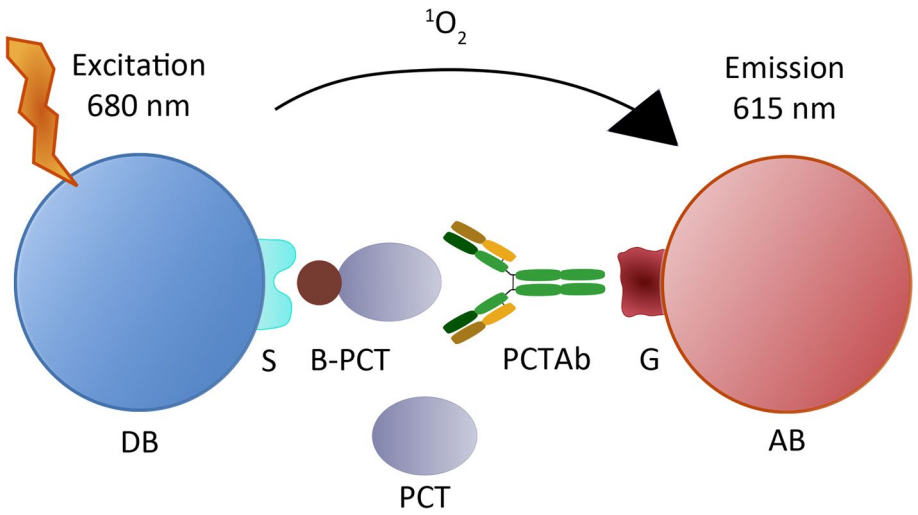

Fig. 2 Schematic picture of the AlphaLISA reaction for procalcitonin detection. DB, donor bead; S, streptavidin; B-PCT, biotinylated procalcitonin; PCT, procalcitonin of the sample; PCTAb, Anti-procalcitonin polyclonal antibody; G, protein G; AB, acceptor bead

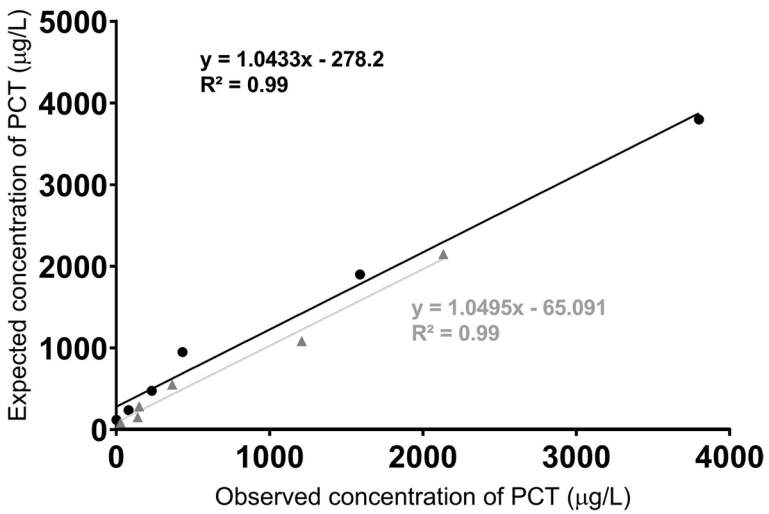

Fig. 3 Linearity of dilution of two saliva samples with a high concentration of procalcitonin (µg/L). The obtained salivary procalcitonin concentrations are represented in X-axis, and the expected salivary procalcitonin concentrations are represented in Y-axis. r^2 = coefficient of determination

were of lower magnitude compared with the LPS group. For example, the pigs with the LPS administration had a mean 6.35-fold increase at T24 compared to TB, whereas at T24, the pigs with the turpentine oil administration had a mean 1.64-fold increase compared to TB. These increases in PCT in non-septic inflammatory conditions have been described in humans in conditions like severe burns, trauma, or major surgery. These increases are usually of less magnitude [28], similarly to what occurs in our study, and therefore, in human medicine, the use of cut-off points that help differentiate diseases caused by sepsis and non-septic inflammatory conditions is standardised [29].

At the farm level, the piglets with meningitis in our study had a mean 3.53-fold increase in PCT concentrations compared to healthy piglets. The magnitude of increases in PCT obtained in our study in sepsis is consistent with previous studies performed in human saliva. For example, a 3.45-fold increase in salivary PCT was observed in exacerbations of bacterial origin

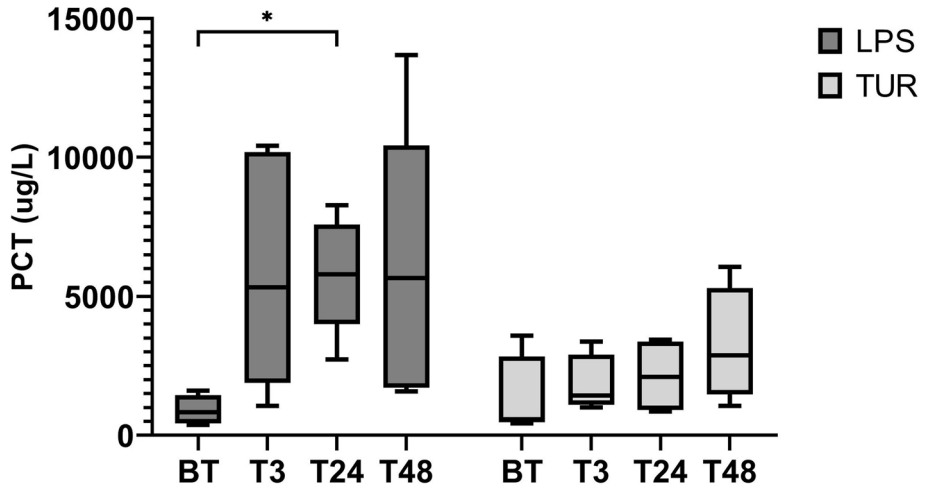

Fig. 4 PCT concentrations (µg/L) at the evaluated times in the LPS and turpentine oil. BT = basal time; T3, T24 and T48 = 3, 24 and 48 h after the injections of LPS and oil-turpentine in both groups. Graphs show medians (line within box), 25th and 75th. percentiles (boxes), min and max values (whiskers) and individual values (points). Asterisks indicate statistically significant differences (*$P < 0.05$)

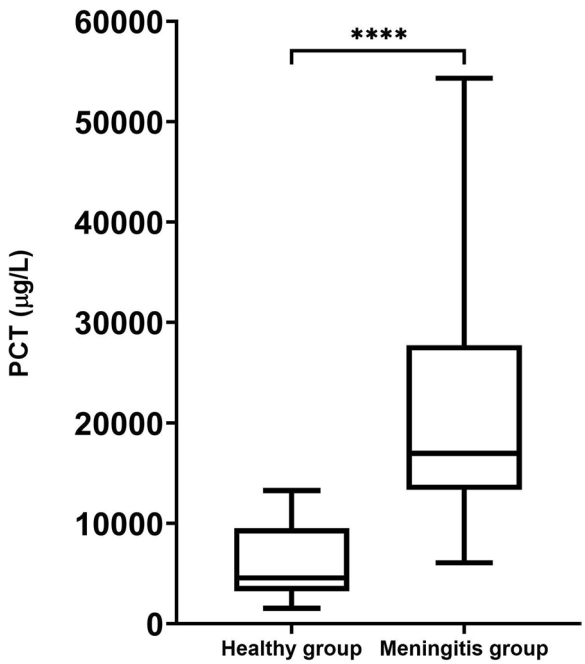

Fig. 5 PCT concentrations (µg/L) in the pigs with meningitis compared with the healthy group. Graphs show medians (line within box), 25th and 75th. percentiles (boxes), min and max values (whiskers) and individual values (points). Asterisks indicate statistically significant differences (**** = $P < 0.0001$)

in Chronic Obstructive Pulmonary Disease [13]. In our study, the magnitude of increase of PCT in piglets with meningitis compared to healthy ones was lower than in the LPS group. These variations could be related to the differences in severity and duration over time of the two conditions. They could also be influenced by differences based on age, as in human medicine, PCT average concentrations are higher in neonates [30], and in our study, the piglets from the healthy/meningitis group were younger than the pigs from the LPS model. Therefore, reference ranges for different pathologies and ages should be established in the future in the case of PCT in saliva. Also, PCT levels in saliva could be influenced by the health status of the farm, a factor that should be studied in more detail in future research.

The main limitation of this report is the number of animals used; consequently, this should be considered a pilot study. In addition, although the target of the study was to validate the method in a non-invasive sample that did not generate stress on the animals, another limitation would be the lack of comparison with other biological samples such as serum. A correlation was reported between procalcitonin in saliva and serum from humans [31], but there is no data in the pig. Therefore, further research would be of interest to validate this assay in other potential samples. In addition, a more significant sample and a more extensive range of pathologies at the farm level are necessary to set optimal cut-offs points, ideally at different ages, to differentiate between animals with and without sepsis. Another limitation of this study is that it cannot be ensured that in our work conditions, the streptococcal meningitis was not accompanied by other concomitant pathologies that can occur in this disease [32].

Conclusions

A precise and accurate assay has been developed to quantify PCT in the saliva of pigs. This assay was applied to the saliva of pigs with experimentally induced sepsis and non-septic inflammation, and the increases were higher in the septic model. The assay also detected higher values of PCT in the saliva of piglets with meningitis compared to healthy piglets. Therefore, this assay could be potentially used as a tool for the non-invasive detection of sepsis in pigs, which is currently a topic of high importance due to antibiotic use restriction.

Methods

Development and optimisation of the assay for PCT measurement

Antibody production

Antibodies were produced according to standard protocols (University of California Berkley Animal Care and Use Committee, 2009) in a New Zealand rabbit (female, 2.5 kg, 3-months old) supplied by the commercial farm Granja San Bernardo (Navarra, Spain). The rabbit was immunised using 100 µg of porcine PCT (Biovendor, RD572451100) as an antigen, diluted in NaCl and emulsified in Freund's adjuvant (complete in the first immunisation, incomplete in the booster ones) in a total of 0.2 ml subcutaneously [33]. A week after each immunisation, blood was collected via the auricular vein of the rabbit, and serum was screened through ELISA to evaluate the antibody titration. After the final blood collection, the rabbit was anaesthetised with intramuscular xylazine at a dose of 3–9 mg/kg. Then, when the rabbit reached unconsciousness, it was euthanised by barbiturate overdose through intravenous administration of sodium pentobarbital at a dose of 150 mg/kg.

Antibody purification

To avoid interferences of the antibodies with other compounds, they were purified with an automated liquid chromatography system (ÄKTA pure, GE Healthcare Life Sciences), passing the rabbit serum through a HiTrap protein G HP affinity column according to the manufacturer's instructions (GE Healthcare Life Sciences, Munich, Germany).

PCT biotinylation

PCT was biotinylated with the commercial kit EZ-Link™, Micro Sulfo-NHS-Biotin, No-Weight™ Format (Thermo Scientific, USA) with a 50-fold molar excess following the manufacturer's instructions.

Development and optimisation of AlphaLISA method

AlphaLISA technology (PerkinElmer, Inc., MA, USA) allows the development of amplified luminescent proximity homogeneous assays that provide several advantages over other similar assays, such as the no need to wash the plate or the use of minimal sample quantities. An indirect competitive assay was developed for PCT measurement, which can be performed in 96-well plates (PerkinElmer, Inc., MA, USA) with a total volume of 50 uL per well. In order to optimise assay conditions, different concentrations of all components were evaluated. The performance of each combination was tested with a constant amount of procalcitonin (1000 ng/ml) and assay buffer used as a blank. Then, the magnitude of signal change (expressed as counts in AlphaLISA assays), the maximum signal obtained and the buffer/protein ratio were evaluated with each condition. The combinations that were tested included 0, 0.3, 3, 4.5 and 6 nM of biotinylated PCT; 10 and 15 nM of polyclonal antiPCT antibody; 5, 10, 15 and 20 µg/ml of Donor beads coupled to streptavidin; and 5, 10, 15 and 20 µg/ml of Acceptor beads coupled to protein G (PerkinElmer, Inc., MA, USA). In addition, several samples with high and low concentrations of procalcitonin were diluted ranging from 1:2 to 1:16 to assess which dilution showed best linearity. As a standard, a commercial porcine PCT (Biovendor R&D, Brno, Czech Republic) was used, and the curve was conducted with concentrations ranging from 10 to 10000 ng. Finally, the performance of three different buffers (PBS, alpha buffer and universal buffer, the last ones from PerkinElmer, Inc., MA, USA) was tested with the standard and several samples. Results were expressed in µg/L.

Analytical validation of the AlphaLISA method

Imprecision

Imprecision was assessed through intra and inter-assay coefficients of variation (CVs), calculated as the standard deviation divided by the mean of the values of the different replicates multiplied by 100. The intra-assay imprecision was calculated by measuring five replicates of samples with a high, medium, and low concentration of PCT at the same time. The assessment of the inter-assay imprecision was performed by the measurement of five aliquots of each saliva sample that were stored at -80ºC, and each one was analysed in duplicate along five different days. All the samples used in the analytical validation were obtained from the LPS experimental model described in this article.

Accuracy

Accuracy was tested by assessing the linearity of serial sample dilution. Also, the matrix effect was tested

through spike and recovery tests as previously reported [34, 35]. Two samples with a high PCT concentration were serially diluted from 1:4 to 1:128 with the assay buffer for the linearity assessment. The recovery experiment was performed by adding different concentrations of porcine PCT standard (10000, 5000, 3000, 1000, 300 and 10 ng) to a diluted (1:4) porcine saliva sample with a low concentration of PCT.

Sensitivity

Sensitivity was assessed by the limit of detection (LOD) and the lower limit of quantification (LLOQ). LOD was calculated as the mean value of 12 replicate PCT determinations of the assay buffer plus three standard deviations (SD). In contrast, LLOQ was evaluated by serially diluting a porcine saliva sample with the assay buffer and analysing five replicates of each dilution. Then, CVs of each dilution were calculated, and LLOQ was set on the lowest PCT concentration that could be repeatedly measured with a 20% CV or lower.

Changes in PCT in an LPS experimental model

Animals

Growing male pigs [(*Sus scrofa domesticus) (Large White)]* in the mid-fattening period from the Experimental Farm of the University of Murcia (Murcia, Spain) were used to perform the experimental model. On this farm, pigs are kept from birth until they are sent to the slaughterhouse at about 24 weeks old. At the moment of sampling, pigs were 14 weeks old and had a median weight of 51.5 kg (interquartile range 48–53 kg). Pigs were given *ad libitum* access to a nutritionally balanced diet and water and were housed with a minimum space of 0.65 m^2 per animal (Council Directive 2001/88/CE of 23 October 2001 amending Directive 91/630/CEE concerning minimum standards for the protection of pigs) and an average temperature of 24 ± 2 °C.

Groups and sample collection

A total of nine animals were chosen at random by convenience sample and were divided into two separate groups for the experimental model. Animals were adapted to experimental conditions (groups, housing, diet, and ambient temperature) for one week before starting the experiment. All animals and samples obtained were appropriately identified to avoid potential confounders. No prior potential animal exclusion criteria were established, and there were no later exclusions from the study. All participants were aware of the location of the two groups during all phases of the experiment. The first group ($n=5$) was administered LPS from *Escherichia Coli (LPS; O55:B5, Sigma-Aldrich)* reconstituted in sterile saline solution in a single dose of 30 ug/kg per animal by intramuscular route [36]. In the second group ($n=4$), the animals were treated with a total of 8 mL subcutaneous injections of turpentine oil (oil of turpentine purified, Sigma–Aldrich), 4 mL in each front flank per animal, as previously described [37].

The administration of the compounds was performed between 8–9 am, and based on the time of the injection of each animal, 4 sample collection times were established: BT (basal time, 24 h before the LPS and turpentine oil injections), used as a control sample; and T3, T24 and T48 (3, 24 and 48 h after the respective intramuscular injections). At each time, saliva samples from all the animals were collected. Additionally, rectal temperature was measured 6 h after administration of both compounds.

Saliva was collected using a sponge clipped to a flexible thin metal rod approximately 20 cm in length. Pigs were allowed to chew on the sponge until thoroughly moist, and then, the sponges were introduced in *Salivette* tubes (Sarstedt, Aktiengesellschaft & Co. D-51588 Nümbrecht, Germany). All samples were kept refrigerated until arrival at the laboratory, where the *Salivette* tubes were centrifuged at 3000 *g* and 4ºC for 10 min to obtain saliva. Then, samples were transferred into Eppendorf tubes and stored at -80ºC until analysis.

Changes of PCT in meningitis

Animals

Weaning pigs [(*Sus scrofa domesticus) (Large White)*] from 6 to 9 weeks old from a commercial farm located in the same geographical area as the University of Murcia (Region of Murcia, south-eastern Spain) were chosen at random by convenience sample. Two groups were established, integrated by clinically healthy pigs ($n=11$) belonging to one pen and pigs diagnosed with meningitis ($n=20$) belonging to another pen. All animals and samples obtained were appropriately identified to avoid potential confounders. The animals with meningitis had clinical symptomatology compatible with this disease [32] and had been recently positive to *Streptococcus suis* in bacteriological cultures performed in blood agar plates following standard procedure [38]. After the study, the animals were kept in the farm under the standard production management. No prior potential animal exclusion criteria were established. There were no later exclusions from the study. All participants were aware of the location of the two groups during all phases of the experiment.

Sample collection

Saliva was collected, processed and analysed as described in the previous subsection Groups and sample collection.

Statistical analysis

Descriptive statistics and linear regression equations were calculated using routine descriptive statistical procedures and computer software (Excel 2016, Microsoft). Statistical analysis and graphs were performed using RStudio software (RStudio Team (2019). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/) and Graph Pad software (GraphPad Prism, version 9 for Windows, Graph Pad Software Inc., San Diego, USA).

The LPS and turpentine oil experiment data were evaluated for normality of distribution using the Shapiro–Wilk test and showed a non-normal distribution; therefore, data were log-transformed and then analysed with a linear mixed model followed by a multiple comparisons test. The data from the healthy piglets and piglets with meningitis were evaluated for normality of distribution using the Shapiro–Wilk test and showed a non-normal distribution; therefore, data were log-transformed and evaluated with an unpaired-two sample t-test to evaluate differences between both groups. Results were reported as mean ± SD of results expressed as µg/L of PCT and represented in box and whiskers plots in Figures. The alpha level for determination of significance was 0.05.

Abbreviations

PCT: Procalcitonin; LPS: Lipopolysaccharide; CVs: Coefficients of variation; LOD: Limit of detection; LLOQ: Lower limit of quantification; SD: Standard deviation.

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We thank Raquel Solano Martínez from Cefu S.A. for her assistance in sampling the groups of healthy piglets and piglets with meningitis.

Authors' contributions

JJC, SM and FT designed the study and contributed in the implementation of the research. MJL and SM performed the development and validation of the PCT assay. MJL, DE, SM and GR performed the LPS and turpentine oil model experiment and took the saliva samples. MJL analysed the data, drafted the manuscript and designed the graphics. JJC, FT and EGM were major contributors to the analysis of the results and to the writing and revising of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The Ethical Committee on Animal Experimentation (CEEA) of the University of Murcia approved the research protocol in this study with approval number CEEA 563/2019, according to the European Council Directives regarding the protection of animals used for experimental purposes. Also, this study complies with ARRIVE guidelines for the care and use of animals.

Consent for publication

Not applicable.

Competing interests

J. J. Cerón is a Senior Board Member of the journal.

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Publisher's Note

CONCLUSIONS

1. The biomarkers with a potential in the diagnosis and monitoring of sepsis can be classified into three main categories: (1) acute phase proteins and cytokines, which have been traditionally used in veterinary medicine for evaluation of inflammation; (2) PCT, PSE, and other more recent proteins that are more specific of bacterial infections; and (3) other markers that can provide complementary information evaluating endothelial damage, organic dysfunction, or alterations in the coagulation system. It is essential to continue testing the ability and applications of these biomarkers to reduce the negative consequences of sepsis in farms.

2. In the proteomic studies, saliva and serum showed different patterns in response to septic inflammation in a model induced by LPS and meningitis due to *Streptococcus suis.* That could indicate that both types of samples could provide complementary information about the physiologic mechanisms of sepsis. In addition, changes in proteins in saliva were found in other septic diseases, such as diarrhoea caused by *Escherichia coli.* The changes that some of the proteins showed in the proteomic studies were validated with spectrophotometric assays, which also showed changes in line with the proteomic results in a larger population of pigs.

3. Sepsis produces changes in salivary analytes related to stress, redox status, inflammation, and muscle damage, which opens the possibility of using them as potential biomarkers for this process in the pig. In this line, further studies are necessary to define the ability and applications of these biomarkers to diagnose and monitor sepsis, as well as establish accurate cut-off values.

4. Procalcitonin was measured for the first time in porcine saliva and showed higher increases in pigs in different septic conditions, such as in an LPS-induced model, *Streptococcus suis* meningitis, and a tail-biting outbreak, than in non-septic inflammatory processes like a turpentine-induced model.

RESUMEN

La sepsis es una afección grave y potencialmente mortal caracterizada por una respuesta inflamatoria sistémica desencadenada por un agente infeccioso, que finalmente puede conducir a la disfunción orgánica e incluso a la muerte. Las tasas de morbilidad y mortalidad aumentan si la sepsis no se diagnostica y trata bien a tiempo. Además, la caracterización inadecuada del agente causal de la sepsis puede conducir a un uso incorrecto de los antibióticos, lo que incrementa el desarrollo de resistencias a estos medicamentos (Riedel & Carroll, 2013; Taylor, 2015; Weiss et al., 2015; Smyth et al., 2016; Luppi, 2017). Sin embargo, el diagnóstico de sepsis sigue siendo un desafío: los signos y síntomas clínicos de la sepsis pueden superponerse con los de otras afecciones no infecciosas, lo que dificulta diferenciar y confirmar la presencia de sepsis, y los métodos diagnósticos convencionales, como el hemocultivo, tienen limitaciones, incluyendo retrasos en la obtención de resultados y una alta incidencia de falsos negativos (Gotts & Matthay, 2016; Singer et al., 2016; Jereb et al., 2019).

Para abordar estos desafíos, cada vez es más necesaria la identificación de biomarcadores que permitan la detección temprana y la caracterización del agente causal de la sepsis. En este contexto, los biomarcadores pueden proporcionar información valiosa sobre la presencia y la gravedad de la afección, así como su agente infeccioso desencadenante. En medicina humana, se utilizan de rutina varios biomarcadores para detectar de forma temprana las infecciones bacterianas y guiar en la administración de antibióticos, como la procalcitonina (Pierrakos & Vincent, 2010; Liu et al., 2016; Matur et al., 2017; Sager et al., 2017). En veterinaria, a pesar de que las enfermedades inflamatorias e infecciosas conllevan muchos problemas de salud y pérdidas económicas en las granjas, este campo está poco explorado. Al ampliar nuestro conocimiento de los biomarcadores inflamatorios y de sepsis y mejorar sus técnicas de detección y medición, podemos conducir a una intervención temprana y un tratamiento más adecuado, mejorando en última instancia la salud y el bienestar de los cerdos, y reduciendo el desarrollo de resistencias a los antibióticos y las pérdidas económicas en las granjas. La muestra más conveniente para medir esos biomarcadores sería la saliva, ya que se considera una muestra no invasiva que asegura el bienestar animal y permite la recogida seriada de muestras, incluso en el mismo día, y por personal no capacitado (Cerón, 2019; Wolf et al., 2020).

Esta tesis doctoral busca contribuir al campo del diagnóstico y caracterización de la sepsis en cerdos profundizando y ampliando nuestra comprensión de los biomarcadores inflamatorios, su comportamiento y su relevancia en estos animales.

Los objetivos específicos de esta Tesis Doctoral estuvieron en línea con los avances en biomarcadores de inflamación, especialmente séptica, en saliva de cerdo:

- **Objetivo 1.** Investigación bibliográfica sobre el conocimiento de la sepsis y los biomarcadores actuales más utilizados para diagnosticar y monitorizar este estado patológico en veterinaria. Esta investigación dio lugar al artículo nº 1.

- **Objetivo 2.** Identificación de nuevos biomarcadores potenciales mediante técnicas proteómicas en:
    - Un modelo experimental de inflamación séptica y no séptica mediante la administración de LPS de E. coli y aceite de trementina, respectivamente, en cerdos.
    - Muestras procedentes de cerdos de granjas comerciales con meningitis por *S. suis.*
    - Muestras procedentes de cerdos de granjas comerciales con diarrea por *E. coli.*

  Los resultados de este objetivo están publicados en los artículos nº 2, 3 y 4.

- **Objetivo 3.** Validación y medición de diversos biomarcadores de inflamación, estrés oxidativo, bienestar y daño muscular con potencial aplicación en inflamación séptica, y estudio de sus posibles cambios en sepsis y otras afecciones. Los resultados de este objetivo se publican en los artículos nº 5 a 8 y se someten a publicación en el Experimento 1 en Anexos.

- **Objetivo 4.** Desarrollo y validación de nuevos ensayos para diagnosticar la sepsis: procalcitonina y presepsina. Los resultados de este objetivo se publican en el documento nº 9, y se publicarán en el futuro en dos experimentos descritos en Anexos (Experimentos 2 y 3).

Todos los procedimientos con animales de experimentación en esta Tesis Doctoral se realizaron de acuerdo con el principio de las Tres Erres de Experimentación Animal siguiendo la legislación española (RD53/2013) y europea (Directiva 2010/63/UE). Los

experimentos que requirieron manipulación animal fueron aprobados por el Comité Ético de Experimentación Animal (CEEA) de la Universidad de Murcia, bajo los números de protocolo CEEA 171/2015, 235/2018 y 563/2021. En cuanto a la producción de anticuerpos monoclonales, además de ser aprobados por su correspondiente CEEA, los procedimientos siguieron la normativa europea sobre la producción de anticuerpos monoclonales (ECVAM Workshop 23, 1997). El modelo de transporte de cerdos que se utilizó para evaluar varios biomarcadores se realizó de acuerdo con las recomendaciones descritas en la Directiva 2001/88/CE, 2001 y la Directiva 2001/93/CE, 2001. El protocolo de estudio utilizado en la investigación colaborando con el Centro de Investigación para el Bienestar Animal en Finlandia fue considerado éticamente aceptable por el Comité de Ética de Investigación del Campus Viikki de la Universidad de Helsinki (Declaración 2/2022).

Para alcanzar los objetivos de esta Tesis, **se desarrollaron anticuerpos policlonales y monoclonales.** En el caso de los policlonales, para la procalcitonina se inmunizó a un conejo, y para la presepsina, a una cabra, siguiendo protocolos estándar. En ambos casos, los respectivos sueros conteniendo los anticuerpos policlonales se obtuvieron en repetidas ocasiones. En el caso de los monoclonales, la especie elegida para las inmunizaciones fue en ambos casos, el ratón. Los ratones que desarrollaron mejor respuesta frente a los antígenos se sacrificaron por dislocación cervical, y los linfocitos B de su bazo se sembraron en placas de 96 pocillos y se fusionaron con células de mieloma para producir hibridomas, siguiendo protocolos previos (Yokoyama, 1999). Tanto los sueros con anticuerpos policlonales, como los sobrenadantes obtenidos de los anticuerpos monoclonales, fueron testados con ELISA y Western Blot para confirmar su afinidad con procalcitonina y presepsina, y posteriormente purificados mediante columnas de afinidad.

En cuanto a la **recolección de muestras en los cerdos**, la saliva se obtuvo mediante esponjas de polipropileno sujetas a varillas metálicas flexibles o a fórceps, introduciéndose suavemente en la boca de los cerdos, salvo en aquellos que vinieran voluntariamente a masticar las esponjas. Una vez que las esponjas estaban completamente humedecidas, se colocaban en tubos Salivette. En cuanto a las muestras de sangre, se obtuvieron mediante punción de la vena yugular y se recogieron en tubos lisos de vacío (BD Vacutainer, Franklin Lakes, NJ, EUA). Todas las muestras se mantuvieron a 4-8 °C en un refrigerador portátil hasta su llegada al laboratorio donde el vacutainer y/o los tubos

Salivette se centrifugaron a 3000× g y 4°C durante 10 min para obtener sobrenadante de suero y saliva, respectivamente. Luego, las muestras se transfirieron a tubos Eppendorf y se almacenaron a -80°C hasta el análisis.

En varios de los ensayos de esta Tesis Doctoral se utilizó una **experimental en la que se indujo inflamación séptica y no séptica en cerdos**. Las inducciones se realizaron en 15 cerdos macho en el período de engorde medio. En el primer grupo (*n*=5; grupo control), se administró una inyección de NaCl (2 mL) por vía intramuscular. El segundo grupo (*n*=5; LPS) recibió una dosis única de 30 μg/kg de LPS de *Escherichia coli* mediante inyección intramuscular. En el tercer grupo (*n*=5, grupo TURP), se administraron 8 ml de aceite de trementina purificado a través de dos inyecciones subcutáneas de 4 ml en cada flanco frontal por animal. Se obtuvieron muestras de saliva y sangre pareadas 24 h antes (tiempo basal) de estas administraciones, y 3, 6, 24 y 48 h después de ellas, aunque no todas las muestras se utilizaron para todos los estudios, debido a la cantidad insuficiente de la muestra.

Para el **desarrollo de los ensayos**, se utilizó la tecnología AlphaLISA, que se basa en la química de canalización de oxígeno luminiscente. Los inmunoensayos AlphaLISA pueden diseñarse en una configuración sándwich o competitiva, y tienen sus propias ventajas, como el uso potencial de cantidades mínimas de muestra (el volumen total por pocillo es normalmente de 50 μl) o la ausencia de pasos de lavado.

El ensayo desarrollado para medir la procalcitonina fue un ensayo competitivo indirecto con un anticuerpo policlonal. Para optimizar las condiciones de ensayo, se evaluaron diferentes concentraciones de todos los componentes (acceptor beads, proteína biotinada, anticuerpo, donor beads). El rendimiento de cada combinación se probó con una cantidad constante de estándar con concentración conocida y tampón de ensayo utilizado como blanco. Como estándar, se utilizó PCT porcina comercial, y la curva se preparó con concentraciones que oscilaban entre 10 y 10000 ng. Los resultados se expresaron en μg/L.

Todos los **ensayos utilizados han sido validados,** ya sea en esta Tesis Doctoral, o previamente a ella. La validación en todos los casos se realizó mediante protocolos descritos previamente (Andreasson et al., 2015), evaluando precisión intra e interensayo, exactitud mediante linealidad en muestras con diluciones seriadas y pruebas de

recuperación, y sensibilidad, mediante el cálculo de límite de detección (LOD) y límite inferior de cuantificación.

Para la **identificación de nuevos biomarcadores, se realizaron análisis proteómicos** en saliva de cerdo. Para ello, se utilizaron tanto técnicas proteómicas basadas en gel, como sin gel. En el caso de las técnicas basadas en gel, se realizaron electroforesis SDS-page (1 dimensión, 1DE) y electroforesis bidimensional (2 dimensiones, 2DE). Las bandas y manchas proteicas obtenidas, respectivamente, se analizaron para detectar diferencias significativas entre grupos de animales. Finalmente, las bandas y manchas de interés se digirieron con tripsina y se analizaron mediante técnicas proteómicas sin gel, en este caso mediante análisis de cromatografía líquida de alta resolución y espectrometría de masas (HPLC-MS/MS). Otras muestras de esta Tesis Doctoral se analizaron al completo mediante técnicas proteómicas sin gel, en este caso procesadas mediante etiquetado isobárico con etiquetas de masa en tándem (TMT) y posteriormente analizadas mediante análisis de cromatografía líquida mediante espectrometría de masas (LC-MS/MS).

En cuanto al **análisis de los biomarcadores estudiados en esta tesis,** se han empleado una gran variedad de métodos representativos de estrés, inflamación, estado redox o daño muscular, basados en diferentes técnicas:

- Kits comerciales automatizados en Olympus AU400 (Beckman Coulter): dímero D, sAA, ADA e isoenzimas, ALDOA, CALP, CK, CK-MB, lactato, LDH, AST, ALT, proteínas totales, CUPRAC, FRAS, TEAC, ácido úrico, AOPP, FOX, POX-Act y d-ROMS.
- Kits ELISA: calgranulina c.
- Inmunoensayos quimioluminiscentes automatizados con Immulite 1000 (Siemens Healthcare Diagnostic): troponina I y cortisol.
- Ensayos AlphaLISA: cortisol, oxitocina, haptoglobina.

A continuación, se muestra un resumen de los estudios realizados durante esta Tesis, que permitieron alcanzar los objetivos propuestos: nueve de ellos fueron publicados en revistas científicas de alto impacto y reconocidas internacionalmente, y tres se han llevado a cabo durante la tesis y están o van a estar próximamente en trámites de publicación.

OBJETIVO 1: Revisión bibliográfica sobre el conocimiento de la sepsis y los biomarcadores actuales más comunes utilizados para diagnosticar y monitorizar este estado patológico en medicina veterinaria. Este objetivo se plasmó en el artículo nº 1.

En el **artículo nº 1** se realizó una revisión bibliográfica para agrupar el conocimiento actual de biomarcadores utilizados en medicina veterinaria para el diagnóstico de la sepsis, así como de potenciales biomarcadores que podrían utilizarse en el futuro, y discutir posibles avances en este campo. La sepsis es un síndrome clínico complejo desencadenado por una respuesta inflamatoria del huésped a una infección. Por lo general, es complicado de detectar y diagnosticar, y tiene graves consecuencias en la salud humana y veterinaria, especialmente cuando el tratamiento no se inicia a tiempo. Además, un diagnóstico adecuado podría no solo mejorar los tratamientos y las posibles consecuencias de la sepsis, si no también reducir el mal uso de los antibióticos, algo esencial para luchar contra la resistencia a estos medicamentos. Esto es un problema importante en los animales de granja, ya que los antibióticos se han administrado tradicionalmente de forma masiva, pero ahora se están restringiendo cada vez más. Cuando se sospecha de sepsis en animales, los biomarcadores que han sido tradicionalmente más utilizados han sido las proteínas de fase aguda como la proteína C reactiva, la amiloide A sérica y la haptoglobina, pero sus concentraciones pueden aumentar en otras afecciones inflamatorias. Por ello, existen otros biomarcadores más prometedores para detectar la sepsis actualmente, entre los que se encuentran la procalcitonina y la presepsina, pero hay poca información sobre la aplicación de estos biomarcadores en especies veterinarias. En esta revisión, se desarrollan todos estos puntos.

OBJETIVO 2: Exploración de nuevos biomarcadores potenciales utilizando técnicas proteómicas en tres situaciones: 1) un modelo experimental de inflamación séptica y no séptica mediante la administración de lipopolisacáridos (LPS) de *E. coli* y aceite de trementina, respectivamente, a cerdos; 2) muestras de cerdos de explotaciones comerciales con meningitis por *S. suis*, y 3) muestras de cerdos de explotaciones comerciales con diarrea causada por *E. coli*. Los resultados de este objetivo dieron lugar a los artículos nº 2-4.

En el **artículo nº 2,** el objetivo fue evaluar los posibles cambios en el proteoma salivar y sérico de cerdos en el modelo de LPS y trementina. El estudio de los cambios que pueden ocurrir en saliva y suero en sepsis puede contribuir a una mejor comprensión de los mecanismos fisiopatológicos implicados en el proceso y también a descubrir posibles biomarcadores que pueden ayudar en su diagnóstico y seguimiento. Tras el análisis con técnicas proteómicas, se validó uno de los biomarcadores con mayor potencial observado (aldolasa A, ALDOA) en un método automatizado.

En la saliva, se encontraron dieciocho proteínas salivares expresadas de forma diferente en la condición de sepsis y nueve en la inflamación no séptica. Entre estos, se encontraron incrementos significativos en ALDOA y serpina B12 solo en el modelo de sepsis. Los cambios en la aldolasa A se validaron en una población más grande de cerdos con sepsis debido a la infección por *S. suis*. En suero, 30 proteínas se expresaron de forma diferente en el grupo de sepsis y 26 proteínas en el grupo no séptico, y la mayoría de las proteínas que cambiaron en ambos grupos estaban relacionadas con inflamación no específica. En la saliva de los animales sépticos se activaron algunas vías específicas, como el proceso metabólico del compuesto organonitrogenado y el transporte de lípidos, mientras que, en el suero, una de las principales vías activadas fue la regulación de la secreción de proteínas. En general, la saliva y el suero mostraron diferentes variaciones del proteoma en respuesta a la inflamación séptica y podrían proporcionar información complementaria sobre los mecanismos fisiopatológicos que ocurren en esta condición. Además, la aldolasa A salivar se constituye como un potencial biomarcador de sepsis en saliva de cerdo.

En el **artículo nº 3**, el objetivo fue investigar los posibles cambios en el perfil proteómico salivar y sérico de lechones con meningitis producida por *Streptococcus suis*, patógeno que causa una alta mortalidad y morbilidad en las granjas porcinas y tiene un potencial zoonótico cada vez mayor en todo el mundo. El análisis del proteoma de la saliva sería potencialmente útil para comprender mejor los cambios fisiopatológicos y para encontrar nuevos biomarcadores que permitan diagnosticar y monitorizar la infección por *S. suis.* Se utilizó el enfoque proteómico LC-MS/MS TMT para analizar muestras de saliva y suero de 20 lechones machos: 10 con meningitis y 10 sanos.

En saliva, se encontraron 11 proteínas con mayor y 10 con menor abundancia relativa en lechones con meningitis. Las proteínas con mayor abundancia relativa fueron

metavinculina (VCL) y desmocolina-2 (DSC2). La adenosina desaminasa (ADA) fue seleccionada para su validación mediante un ensayo automatizado espectrofotométrico disponible comercialmente, y pudo diferenciar de forma excelente entre cerdos sanos y cerdos con meningitis por *S. suis.* En el suero, los cambios más sobresalientes ocurrieron en una serpina y en haptoglobina (Hp). En saliva y suero, el mayor número de proteínas con abundancia alterada estaban relacionadas con las vías plaquetarias y neutrofílicas. En general, la meningitis causada por *S. suis* dio lugar a cambios específicos del proteoma en la saliva y el suero, reflejando diferentes mecanismos fisiopatológicos en cada fluido biológico, y proponiendo nuevos biomarcadores potenciales para esta infección.

En el **artículo nº 4,** el objetivo fue identificar cambios en el proteoma salivar de cerdos con diarrea causada por *Escherichia coli,* bacteria que representa la principal causa de diarrea en cerdos. Se recolectaron muestras de saliva de 10 cerdos con esta enfermedad y 10 controles sanos. Se realizaron electroforesis en gel SDS-PAGE (1DE) y bidimensional (2DE), y se identificaron bandas y manchas de proteínas significativamente diferentes mediante espectrometría de masas. Para la validación, se midió la adenosina desaminasa (ADA) en 28 cerdos sanos y 28 enfermos. En 1DE, se observaron aumentos en las bandas de lipocalina e IgA en cerdos enfermos, mientras que las bandas que incluían proteína de unión a olores (odorant binding protein) y proteína inducible por prolactina (prolactin-inducible protein) presentaron menores concentraciones. Los resultados de la electroforesis 2DE mostraron que la saliva de animales con *E. coli* presentó mayores niveles de expresión de lipocalina, ADA, IgA y péptidos de albúmina, estando también la actividad de ADA aumentada en los cerdos enfermos en el estudio de validación. Las manchas proteicas que contenían alfa-amilasa, anhidrasa carbónica VI y albúmina entera disminuyeron en animales enfermos. En general, se observó que los cerdos con diarrea causada por *E. coli* tuvieron cambios en las proteínas de su saliva relacionados con diversos mecanismos fisiopatológicos como la inflamación y la función inmune y que podrían ser biomarcadores de esta enfermedad.

OBJETIVO 3: Validación y medición de diversos biomarcadores de inflamación, estrés oxidativo, bienestar y daño muscular con potencial aplicación en inflamación séptica, y estudio de sus posibles cambios en sepsis y otras afecciones. Los resultados de este objetivo se tradujeron en los artículos nº 5-8 y en el Experimento 1 en Anexos.

En el **artículo nº 5,** el objetivo fue evaluar si las concentraciones de dímero D podrían medirse en saliva de cerdo, y si sus concentraciones cambiarían tras un estímulo de estrés agudo. El dímero D es un péptido derivado de la degradación de los coágulos sanguíneos que se encuentra en el suero y que no se había evaluado previamente en la saliva de ninguna especie veterinaria. Para llevar a cabo el primer objetivo del estudio, se realizó una validación analítica completa de un ensayo inmunoturbidimétrico disponible comercialmente. Para el segundo objetivo, se indujo un modelo experimental de estrés agudo en 11 cerdos basado en una técnica de sujeción mediante inmovilización nariz-trampa durante 1 minuto. Posteriormente se recogieron muestras de saliva en diferentes momentos y se evaluaron el dímero D, la alfa-amilasa salivar (sAA) y el cortisol para evaluar los cambios en sus concentraciones después del estrés.

El ensayo automatizado del dímero D mostró una precisión, reproducibilidad y sensibilidad adecuadas. En el modelo de estrés, se produjo un aumento significativo ($p<0.05$) en el dímero D salivar tras 15 min del estímulo estresante, y una correlación positiva entre el dímero D y sAA ($r=0.51$; $p<0.001$). Estos resultados indican que el dímero D se puede medir en la saliva porcina con un método automatizado y sugieren que su concentración puede verse influenciada por condiciones estresantes.

En el **artículo nº 6**, se evaluaron los cambios producidos en biomarcadores de estado redox en saliva de cerdos con una inflamación séptica inducida experimentalmente mediante la administración de lipopolisacárido (LPS) de *Escherichia coli,* y con una inflamación aséptica inducida mediante la inyección de trementina. Para ello, se midieron en saliva la capacidad antioxidante reductora cúprica (CUPRAC), la capacidad reductora férrica de la saliva (FRAS), la capacidad antioxidante equivalente de Trolox (TEAC), los productos proteicos de oxidación avanzada (AOPP), la oxidación de xilenol ferroso (FOX), la actividad del peróxido (POX-Act) y los compuestos reactivos derivados del oxígeno (d-ROM). Las tomas de saliva en el grupo LPS, trementina y control (inyectados con suero salino) fueron recogidas antes y después de 3, 6, 24 y 48 horas tras los respectivos tratamientos.

Se vieron mayores concentraciones de AOPP, POX-Act y d-ROM en el grupo de LPS que en el control, de 3 h a 24 h tras la inoculación. CUPRAC, FRAS y TEAC mostraron mayores niveles en el grupo de LPS que en el grupo control a las 24 h. Todos

estos cambios fueron de mayor magnitud que los que ocurrieron en el grupo de trementina. En conclusión, nuestros hallazgos revelan que la sepsis produce cambios en analitos salivares del estado redox, lo que abre la posibilidad de utilizarlos como biomarcadores en esta especie.

En el **artículo nº 7** se evaluaron oxitocina y procalcitonina (PCT), dos analitos representativos del bienestar y la salud animal respectivamente, en un brote espontáneo de mordeduras de colas en cerdos. Ambos biomarcadores tienen potencial para su uso en cerdos y se pueden medir a partir de la saliva, lo que permite el muestreo en la granja con un impacto mínimo en los animales. Se seleccionaron cerdos en fase de crecimiento en una granja comercial pertenecientes a tres grupos fenotípicos diferentes: cerdos control de cuadras control (CC, *n*=30), cerdos control -situados dentro de cuadras en las que había brote de mordeduras de cola- (CTB, *n*=10) y cerdos con lesiones en la cola de cuadras con brote de mordedura de cola (LTB, *n*=27). Se recogió una muestra de saliva de cada cerdo y se analizaron una serie de biomarcadores relacionados con el estrés, la infección, la inflamación y la activación inmune (oxitocina, PCT, cortisol, ADA y Hp).

La oxitocina tendió a estar más alta en cerdos CC que en cerdos LTB, mientras que la procalcitonina tendió a ser más alta en el grupo LTB que en cerdos CC, lo que sugeriría una relación entre sepsis y estrés. Además, en estudios previos se han observado aumentos de oxitocina en animales enfermos con situaciones de compensación para inhibir la inflamación (Işeri et al., 2005). Por tanto, esta hormona podría jugar también un papel importante en la modulación inmune de eventos asociados con la sepsis y ayudando a limitar el daño orgánico (Sendemir et al., 2013).

En el **artículo nº 8,** el objetivo fue validar un ensayo automatizado para la medición de calprotectina (CALP, S100A8/A9) en saliva de cerdo. CALP es un dímero de S100A8 y S100A9 que está involucrado en la inflamación y tiene una amplia gama de funciones proinflamatorias, como la producción de citoquinas y la regulación de la adhesión de leucocitos, la migración y la fagocitosis. En los seres humanos, esta proteína se puede medir en heces, suero y saliva y se utiliza como biomarcador de inflamación y sepsis, pero actualmente no hay métodos para su medición en saliva de cerdo. Además de la validación del método, también se evaluó el biomarcador en tres situaciones diferentes: según la hora del día de la toma (se utilizaron muestras de un estudio previo en las que se

tomaron muestras de saliva de los mismos animales y en el mismo día a las 8 a.m., 12 a.m., 4 p.m., y 8 p.m.), en la experimental de LPS y turpentina descrita en artículos previos en esta tesis, y en un modelo de estrés basado en el transporte de los animales desde la granja a un matadero comercial y su posterior tiempo de espera de 4 horas en el matadero.

En la validación, el ensayo validado mostró precisión y exactitud. CALP en saliva medida por este ensayo mostró una tendencia a ir disminuyendo durante el día, siendo estos cambios significativos a las 12 y 8 p.m. comparado con los valores a las 8 a.m. También mostró aumentos significativos en la saliva de los cerdos 48 horas después de la administración de lipopolisacárido (LPS), y mostró un aumento, aunque con aumentos de menor magnitud, en el modelo de estrés (T4 con respecto a T0).

En el **Experimento nº 1** (actualmente presentado para su posible publicación), se planteó la hipótesis de que la saliva podría reflejar cambios en una infección producida por *Streptococcus suis (S. suis)* en diferentes biomarcadores relacionados con estrés, inflamación, estado redox y daño muscular, y que estos biomarcadores podrían estar relacionados con la gravedad de la enfermedad. Para este estudio, se recolectó saliva de un total de 56 cerdos en crecimiento de una granja fueron seleccionados como cerdos infectados (n=28) y cerdos sanos (n=28). En estas muestras se midió un panel de biomarcadores representativos de los estados ya mencionados, incluyendo cortisol, alfa-amilasa (sAA) y oxitocina (OXT) como biomarcadores de estrés; haptoglobina (hp), proteínas totales, calprotectina (CALP, S100A8-A9) y calgranulina C (S100A12) como indicadores de inflamación; capacidad reductora férrica de la saliva (FRAS) y productos proteicos de oxidación avanzada (AOPP) como biomarcadores de estado redox; creatina cinasa (CK), creatina-cinasa banda miocárdica (CK-MB), troponina I, lactato, lactato dehydrogenasa (LDH), aspartato aminotransferasa (AST) y alanina aminotransferasa (ALT) para evaluar daño muscular, para evaluar inflamación y sepsis. Además, también se midieron tres proteínas relacionadas con el sistema inmune, inflamación y sepsis (ADA, PCT y ALDOA) cuyo aumento en saliva de cerdos con infección por *S. suis* había sido confirmado en estudios previos de esta tesis doctoral.

Los resultados mostraron aumentos en los biomarcadores relacionados con el estrés (sAA y OXT), la inflamación (Hp, proteínas totales, CALP y calgranulina c), y el daño muscular (CK, CK-MB, troponina I, lactato, AST y LDH). También se observó un aumento del ADA, PCT y ALDOA en los animales enfermos, tal y como se había descrito

anteriormente. El grado de gravedad de la enfermedad indicó una correlación positiva significativa con las concentraciones totales de proteínas, AST, ALDOA y AOPP.

En conclusión, la infección por *S. suis* causó variaciones en los analitos relacionados con estrés, inflamación, estado redox y daño muscular en saliva de cerdo, que podrían considerarse potenciales biomarcadores potenciales para esta enfermedad.

OBJETIVO 4: Desarrollo y validación de nuevos ensayos para diagnosticar la sepsis: procalcitonina y presepsina. Este objetivo dio lugar al artículo nº 9, y a los Experimentos 2 y 3 de Anexos.

En el **artículo nº 9**, el objetivo fue desarrollar un método específico para la medición de PCT porcina, y explorar si este biomarcador podría medirse en saliva de cerdo y si su concentración cambia en sepsis. PCT es un biomarcador ampliamente utilizado en sepsis en medicina humana y puede tener aplicaciones potenciales en el campo veterinario, pero la falta de métodos diagnósticos animales lo dificulta. Por lo tanto, se desarrolló y validó un ensayo específico, y se evaluaron los cambios en la concentración de PCT en dos condiciones: a) en el modelo experimental de sepsis producida por la administración de LPS y trementina, y b) en lechones sanos (n=11) en comparación con lechones con meningitis (n=20), una enfermedad que generalmente implica sepsis y cuyo tratamiento a menudo requiere grandes cantidades de antibióticos en las granjas.

El ensayo mostró una adecuada precisión, exactitud y sensibilidad. En el experimento de LPS, se encontraron concentraciones más altas de PCT después de 24 h en los animales inyectados con LPS en comparación con los tratados con aceite de trementina. Además, los animales con meningitis tenían concentraciones más altas de PCT que los cerdos sanos.

Según estos resultados, este ensayo podría ser utilizado como herramienta para la detección no invasiva de sepsis en cerdos, que actualmente es un tema de gran importancia debido a la restricción del uso de antibióticos.

En el **Experimento nº 2** (planificado para ser presentado para su posible publicación), el objetivo fue desarrollar y validar nuevos ensayos con anticuerpos monoclonales para la medición de procalcitonina en saliva de cerdos, y comparar la sensibilidad y especificidad de estos con un ensayo previamente validado, basado en un anticuerpo

policlonal. Los anticuerpos monoclonales y policlonales tienen características distintivas que confieren a cada uno de ellos diferentes ventajas y, por lo tanto, diferentes métodos podrían permitir detectar de manera diferente las proteínas en las muestras. Actualmente, no existe un anticuerpo monoclonal comercial contra la procalcitonina porcina, y existe incertidumbre sobre si diferentes métodos podrían detectar la procalcitonina de una manera diferente que pudiese ayudar en el diagnóstico de la sepsis. Por tanto, el desarrollo de estos anticuerpos monoclonales anti-procalcitonina producidos en el presente estudio sería el primer paso para el desarrollo de nuevos ensayos que podrían ayudar a mejorar el diagnóstico de sepsis en el cerdo. Para producir los anticuerpos monoclonales, se realizó la inmunización de ratones con procalcitonina porcina recombinante, fusión de sus células del bazo con células de mieloma, y selección y clonación de hibridomas, utilizando protocolos previamente publicados.

Se obtuvieron un total de 6 clones celulares que produjeron anticuerpos monoclonales contra la procalcitonina porcina. Los anticuerpos se purificaron con una columna de afinidad y se probaron mediante métodos ELISA y Western blot, mostrando una alta afinidad con la procalcitonina porcina. Actualmente, se está iniciando el desarrollo de métodos utilizando estos anticuerpos monoclonales con la tecnología AlphaLISA utilizada para otros métodos en esta tesis doctoral, y próximamente se validarán los nuevos ensayos mediante precisión intra e interensayo, exactitud mediante linealidad en diluciones seriadas de muestras y pruebas de recuperación, y límites de detección (LOD) y cuantificación (LLOQ). A continuación, se medirá un lote de muestras de saliva de cerdos con diferentes enfermedades inflamatorias e infecciosas y un lote de muestras de saliva de cerdos sanos con los diferentes métodos resultantes para comparar su sensibilidad y especificidad para detectar procalcitonina en las muestras en el diferente estado de salud.

En el **Experimento nº 3** (planificado para ser presentado para su posible publicación), el objetivo fue desarrollar anticuerpos policlonales y monoclonales con una alta afinidad con presepsina porcina (PSE, sCD14). PSE es una proteína estrechamente relacionada con la sepsis, ya que aparece cuando el receptor CD14 del sistema inmune se escinde después de tener contacto con antígenos bacterianos. Sin embargo, no hay un método para medirlos en el cerdo y, por tanto, se desconoce cómo este biomarcador podría ayudar en el diagnóstico de la sepsis. Para ello, se realizó: 1) la inmunización de una cabra con PSE

y las posteriores extracciones sanguíneas para obtener suero con anticuerpos policlonales, y 2) la inmunización de ratones con PSE porcina recombinante, fusión de sus células del bazo con mieloma, y selección y clonación de hibridomas; en todos los casos utilizando protocolos previamente publicados.

Obtuvimos 2 clones celulares que produjeron anticuerpos monoclonales contra la PSE porcina. Los anticuerpos policlonales y monoclonales se purificaron con una columna de afinidad y se testaron mediante ELISA y western blot, mostrando una alta afinidad con la PSE porcina. El desarrollo de estos anticuerpos policlonales y monoclonales anti-PSE permitirá probar la combinación de anticuerpos en diferentes formatos utilizando la tecnología AlphaLISA, y ampliar el número de ensayos para caracterizar la sepsis en el cerdo. Una vez que se desarrollen los métodos, el siguiente paso sería validarlos mediante los procedimientos descritos en los métodos generales. Finalmente, se medirá un lote de muestras de saliva de cerdos con enfermedades inflamatorias e infecciosas y de cerdos sanos con el método resultante para estudiar la capacidad del método para detectar sepsis en cerdos.

Las **conclusiones** obtenidas de esta Tesis Doctoral fueron las siguientes:

1. Los biomarcadores con potencial en el diagnóstico y seguimiento de la sepsis pueden clasificarse en tres categorías principales: (1) proteínas de fase aguda y citoquinas, que se han utilizado tradicionalmente en veterinaria para evaluar la inflamación; (2) PCT, PSE y otras proteínas más novedosas que son más específicas de las infecciones bacterianas; y (3) otros marcadores que pueden proporcionar información complementaria evaluando el daño endotelial, la disfunción orgánica o las alteraciones del sistema de coagulación. Es fundamental seguir probando la capacidad y aplicaciones de estos biomarcadores, para reducir las consecuencias negativas de la sepsis en las granjas.

2. En los estudios proteómicos, la saliva y el suero mostraron patrones diferentes en respuesta a la inflamación séptica en un modelo inducido por LPS y en meningitis causada por *Streptococcus suis*. Esto podría indicar que ambos tipos de muestras podrían aportar información complementaria sobre los mecanismos fisiológicos de la sepsis. Además, se encontraron cambios en las proteínas de la saliva en otras enfermedades sépticas como la diarrea causada por *Escherichia coli*. Los cambios que algunas de las proteínas mostraron en los estudios proteómicos fueron validados con ensayos espectrofotométricos en una

población mayor de cerdos, que también mostraron cambios en la línea de los resultados proteómicos.

3. La sepsis produce cambios en los analitos salivares relacionados con el estrés, el estado redox, la inflamación y el daño muscular, lo que abre la posibilidad de utilizarlos como potenciales biomarcadores de este proceso en el cerdo. En esta línea, son necesarios más estudios para definir la capacidad y aplicaciones de estos biomarcadores para diagnosticar y monitorizar la sepsis, así como para establecer valores de corte precisos.

4. La procalcitonina se ha medido por primera vez en saliva porcina, mostrando mayores incrementos en cerdos en diferentes condiciones sépticas, como en un modelo inducido por LPS, en meningitis por *Streptococcus suis* o en un brote de mordeduras de cola, que lo que aumentó en procesos inflamatorios no sépticos como en un modelo de inducción con trementina.

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ANNEX

The following experiments were performed during this thesis and are currently submitted or planned to be submitted for possible publication.

EXPERIMENT 1

Currently submitted for possible publication

Changes in Salivary Biomarkers of Stress, Inflammation, Redox Status, and Muscle Damage due to Streptococcus suis Infection in Pigs

Streptococcus suis (S. suis) is a Gram-positive bacteria that infects pigs causing meningitis, arthritis, pneumonia, or endocarditis. This increases the mortality in pig farms deriving in severe economic losses. The use of saliva as a diagnostic fluid has various advantages compared to blood, especially in pigs. In this study, it was hypothesized that saliva could reflect changes in different biomarkers related to stress, inflammation, redox status, and muscle damage and that these biomarkers could be related to the severity of the disease.

A total of 56 growing pigs from a farm were selected as infected pigs (*n*=28) and healthy pigs (*n*=28). Results showed increases in biomarkers related to stress (alpha-amylase and oxytocin), inflammation (haptoglobin, total protein, S100A8-A9, and S100A12), and muscle damage (creatine kinase (CK), CK-MB, troponin I, lactate, aspartate aminotransferase, and lactate dehydrogenase). An increase in adenosine deaminase (ADA), procalcitonin, and aldolase in infected animals were also observed as previously described. The grade of severity of the disease indicated a significant positive correlation with total protein concentrations, aspartate aminotransferase, aldolase, and AOPP.

This report revealed that *S. suis* infection caused variations in analytes related to stress, inflammation, redox status, and muscle damage in the saliva of pigs and these can be considered potential biomarkers for this disease.

EXPERIMENT 2

Planned to be submitted for possible publication

Comparison of different assays for the procalcitonin measurements in pigs

Monoclonal and polyclonal antibodies have distinctive characteristics that give each of them different advantages and, therefore, different methods could allow to detect differently the proteins in the samples. Currently, there is no commercial monoclonal antibody against porcine procalcitonin, and there is uncertainty over whether different methods could detect procalcitonin in a different way that could help in the diagnosis of sepsis. The aim of this experiment was to develop and validate new assays with monoclonal antibodies for procalcitonin measurement in saliva of pigs, and compare the sensitivity and specificity of them with a previous polyclonal validated assay. To produce the monoclonal antibodies, it was performed the immunization of mice with recombinant pig procalcitonin, fusion of their spleen cells with myeloma, and hybridoma selection and cloning, using previously published protocols. A total of 6 cell clones that produced monoclonal antibodies against pig procalcitonin were obtained. The antibodies were purified with an affinity column were tested by ELISA and western blot methods, showing a high affinity to pig procalcitonin. The development of these monoclonal anti-procalcitonin antibodies produced in the present study are the first step for the development of new assays that could help to improve the diagnosis of sepsis in the pig.

Currently, we are starting the methods development using these monoclonal antibodies with the AlphaLISA technology used for other methods in this doctoral thesis, and soon the new assays will be validated through intra and inter-assay precision, accuracy with linearity in serial sample dilutions and spike recovery tests, and limits of detection (LOD) and quantification (LLOQ). Then, a batch of saliva samples of pigs with different inflammatory and infectious diseases and a batch of saliva samples from healthy pigs will be measured with the different resultant methods to compare their sensitivity and specificity to detect procalcitonin in the samples in the different health status.

EXPERIMENT 3

Planned to be submitted for possible publication

Validation on an assay for the measurement of presepsin in the saliva of pigs

Presepsin (PSE, sCD14) is a protein closely related to sepsis because it appears when the receptor of the immune system CD14 is cleaved after having contact with bacterial antigens. However, there are no method to measure them in the pig, and then, it is unknown how this biomarker could assist in the diagnostic of sepsis.

The aim of this study was to develop polyclonal and monoclonal antibodies with a high affinity to PSE. To this end, it was performed: 1) the immunization of a goat with PSE and the subsequent blood extractions to obtain serum with polyclonal antibodies, and 2) the immunization of mice with recombinant pig PSE, fusion of their spleen cells with myeloma, and hybridoma selection and cloning; in all cases using previously published protocols. We obtained 2 cell clones that produced monoclonal antibodies against pig PSE. The polyclonal and monoclonal antibodies were purified with an affinity column and were tested by ELISA and western blot, displaying a high affinity to pig PSE. The development of these polyclonal and monoclonal anti-PSE antibodies will make it possible to test the combination of antibodies in different formats using AlphaLISA technology, and to widen the number of assays to characterize sepsis in the pig. Once the methods are developed, the next step would be to validate them with intra and inter-assay precision, accuracy with linearity in serial sample dilutions and spike recovery tests, and limits of detection (LOD) and quantification (LLOQ) calculations. Finally, a batch of saliva samples of pigs with inflammatory and infectious diseases and a of saliva samples from healthy pigs will be measured with the resultant method to study the ability to detect sepsis in swine.